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(54) Title: NEW IMPROVED ACYLTRANSFERASE

(57) Abstract: The invention relates to at least one nucleotide sequence, derived from a nucleotide sequence encoding an acyltransferase polypeptide comprising at least one membrane-spanning region, encoding an improved active membrane independent acyltransferase polypeptide in which at least one amino acid residue of the membrane-spanning region has been deleted and/or substituted as compared to the original acyltransferase polypeptide, wherein the encoded active membrane independent acyltransferase polypeptide, wherein the encoded active membrane independent acyltransferase polypeptide can produce fatty acid esters and/or fatty acid thioesters such as triacylglycerols, diacylglycerols, monoacylglycerols, phospholipids, glycolipids, waxesters, acylated carbohydrates, acylated amino acids, and lysolipids, e.g. lysophospholipid, lysolecithin. Thereby one single acyltransferase can be used for the production of a huge number of products. The invention also relates to means and methods for the production of such an improved active membrane independent acyltransferase and the use of such a membrane independent acyltransferase in industry.

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NEW IMPROVED ACYLTRANSFERASE

FIELD OF INVENTION

The invention relates to at least one nucleotide sequence, derived from a
5 nucleotide sequence encoding an acyltransferase polypeptide comprising at least one membrane-spanning region, encoding an improved active membrane independent acyltransferase polypeptide in which at least one amino acid residue of the membrane-spanning region has been deleted and/or substituted as compared to the original acyltransferase polypeptide, wherein the encoded active membrane
10 independent acyltransferase polypeptide can produce fatty acid esters and/or fatty acid thioesters such as triacylglycerols, diacylglycerols, monoacylglycerols, phospholipids, glycolipids, waxesters, acylated carbohydrates, acylated amino acids, and lysolipids, e.g. lysophosphospholipid, lysolecithin. Thereby one single acyltransferase can be used for the production of a huge number of products. The
15 invention also relates to means and methods for the production of such an improved active membrane independent acyltransferase and the use of such a membrane independent acyltransferase in industry.

BACKGROUND OF INVENTION

20 A phospholipid: diacylglycerol acyltransferase (PDAT) has biochemically been characterised in yeast and plants and a gene, LRO1, encoding the PDAT enzyme was identified in yeast (Dahlqvist et al., 2000, PNAS 97:6487-6492). The enzyme was shown to catalyse the formation of triacylglycerols (TAG) by an acyltransfer from phospholipids to diacylglycerols (DAG). Furthermore, the
25 enzymatic activity was found to be localised in the microsomal fraction. The gene encoding the PDAT enzyme was shown to have sequence homologies to the lecithin: cholesterol acyltransferase (LCAT) gene family. The LCAT enzyme is used for the treatment of LCAT deficiencies, such as arteriosclerosis by increasing the activity of LCAT in serum of the mammal to a level effective to decrease the
30 accumulation of cholesterol (WO9717434). The diet habit used by large groups of people today result in high cholesterol values with all other problems, which follow.

Lipases are enzymes that are primarily responsible for the hydrolysis of glycerolipids such as triacylglycerols. However, it is well known that lipases also under certain conditions in water free systems, can catalyse interesterification
35 (Gandhi, 1997, J Am Oil Chem Soc 74 (6): 621-634). The wide berth for employment in a variety of reactions and broad substrate specificity has rendered the lipases to be very useful in a variety of applications such as production of pharmaceuticals, cosmetics, detergents, foods, perfumery, and other organic synthetic materials. One example is the use of an immobilised lipase for the

synthesis of waxes (US 4826767 and US 6162623). The low stability, low activity or selectivity encountered occasionally with a number of these enzymes have been the chief obstacle hindering a more rapid expansion of industrial lipase technology into new applications on a large scale.

5 Additionally, mass-production of waxes have been performed by culturing microorganisms, together with fatty-acids, wherein acyltransferases present within the microorganism convert the fatty acids into waxesters, such as by using the microorganism *Staphylococcus lentus* (JP 1320989). Another example is the use of *Arthrobacter ceroformans* for the production of waxesters (Koronelli et al., 1979, 10 Vestn. Mosk. Univ. Ser 16, Biol 3:62-64). Other examples are the use of transgenic hosts harbouring a gene encoding an acyltransferase for the production of waxes, as described in WO 9310241 and US 5445947.

Industrial application using the above mentioned lipases as biocatalyst, for the production of a variety of waxesters, is limited to the group of lipases and the 15 restrictions these enzymes have both regarding the products that could be produced and the conditions by which these enzymes are active. For example, the esterification must occur in water free solvents and under reduced pressure.

By the use of microorganisms there are limitations such as the need of several purification steps after the synthesis of the waxesters to be able to remove 20 the microorganism and other impurities, which comes along with the culturing method. There are also difficulties in obtaining high yields of the waxesters. The microorganism may be one that naturally encodes enzymes suitable for the synthesis of waxesters, or a genetically modified microorganism, which by the modification obtains the ability to produce waxesters.

25 Furthermore, the waxesters that can be synthesised today are limited due to the substrate specificity of the enzymes catalysing the wax ester synthesis in these microorganisms. Moreover, these enzymes are integral membrane enzymes, which render it impossible to use such enzymes as biocatalyst in a cell free system such as in an industrial reactor.

30 There is a need for new improved enzymes, which enables the production of variety of fatty acid esters to high yields in cost-efficient industrial processes. Examples of fatty acid esters are structured glycerol fatty acid esters such as triacylglycerols with a specific acyl group at the sn2 positions that differs as compared to that of the outer positions and diacylglycerols with specific acylgroups. 35 Production of fat-soluble fatty acid esters by acylation of water-soluble molecules, such as flavours and vitamins, is another example of desirable fatty acid esters. Other valuable fatty acid esters of interest are waxesters (*i.e.* fatty acids esterified to long chain alcohols), or fatty acid esters of molecules such as carbohydrates and amino acids. A method for the production of such compounds can be achieved by

optimising enzymes that already is used as biocatalyst exemplified by the well-known families of lipases or other membrane independent enzymes. However, in nature many of the enzymes catalysing the transfer of acylgroups are integral membrane proteins. Among the membrane independent acyltransferases present in
5 nature the vast majority catalyses an acyl-CoA dependent reaction. Both these classes of acyltransferases are not suited as a biocatalyst in industrial methods since integral membrane protein are not functioning in cell free systems and acyl-CoA is a to costly substrate. Furthermore, in applications involving enzymes belonging to the lipase family the interesterification is dependent on a water free system. Hence,
10 membrane independent acyltransferases that could use acyl-lipids as acyl donors in industrial methods for the manufacturing of fatty acid esters are limited today and no such enzyme is available which can manufacture several different fatty acid ester and/or fatty acid thioesters, i.e., use a lot of different acyl donors and acyl acceptors.

There are also needs for enzymes to be used to improve the
15 properties of complex raw material. For example within the area of food production, modification of different components such as lipids present in food raw material such as milk cereals, vegetables, eggs, vegetable oils, meat, fish, etc is desirable. Examples of improvements achieved by such modifications are enhanced emulsifying properties, increased shelf life, less off-flavour, etc. For example in
20 many food applications enhanced emulsifying properties are desirable and can be achieved by converting phospholipids (i.e. lecithin) present in the food raw material into lysophospholipids. Lipases are commonly used in such applications resulting in elevated levels of lysophospholipids but also unesterified fatty acids that can result in off-flavours. Conversion of phospholipids into lysolipids without increased
25 amounts of unesterified fatty acids is therefore desirable and can be achieved with acyltransferases that transfer the fatty acid from the phospholipid to an acyl acceptor such as monoacylglycerols, diacylglycerols, alcohols, or any other acyl acceptors present in or added to the raw material.

30 BRIEF DISCLOSURE OF THE INVENTION

Accordingly, in a first aspect the invention relates to one or more nucleotide sequence(s), derived from a nucleotide sequence encoding an acyltransferase polypeptide comprising at least one membrane-spanning region, encoding an
35 improved active membrane independent acyltransferase polypeptide in which at least one amino acid residue of the membrane-spanning region has been deleted and/or substituted as compared to the original acyltransferase polypeptide, wherein the encoded active membrane independent acyltransferase polypeptide can produce lysolipids and fatty acid esters and/or fatty acid thioesters such as

lysophosphospholipid, lysolecithin, triacylglycerols, diacylglycerols, monoacylglycerols, phospholipids, glycolipids, waxesters, acylated carbohydrates and acylated amino acids. Such an improved acyltransferase can be used in a huge number of chemical reactions for the production of a large number of different fatty
5 acid esters and/or fatty acid thioesters, which enables the possibility to in a economic way produce a large amount of a single enzyme which then can be used for several purposes.

Additionally, such an acyltransferase, which is capable of catalysing several reactions, enables the possibility to facilitate the production of a number of fatty
10 acid esters and/or fatty acid thioesters by one single acyltransferase. Such an active membrane independent acyltransferase polypeptide may be used in a bioreactor for the production of desired fatty acid esters or as additive in food raw material for modification of its lipid composition without the need of a microorganism or a lipid membrane for the maintenance of the acyltransferase activity.

15 In another aspect, the invention relates to a nucleotide sequence molecule comprising at least one promoter region which functions in a host, the promoter region is operably linked to at least one nucleotide sequence as described above, which is operably linked to at least one non-translated region which functions in a host.

20 In a further aspect, the invention relates to a method for the production of an active membrane independent acyltransferase polypeptide comprising the steps of providing a host cell and a growth medium preparing a host cell culture, culturing the host cell culture and harvesting the host cell culture and recovering the polypeptide.

25 By providing a nucleotide sequence encoding a membrane independent acyltransferase without the ability to become integrated into a membrane and having the ability to utilise different acyl donors and acyl acceptors, the ability to manufacture acylated products by a sole enzyme (*i.e.* fatty acid esters) is increased.

The membrane independent acyltransferase may be used in applications such
30 as cosmetics, pharmaceuticals, foods, food additives, candles, soaps, detergents, laundries, polymers, coatings, plasticizer, drying oils, lubricants, varnishes, linoleum, printing, inks, textile dyes and surfactants, especially within the area of synthesis of stereo specific isomers, which not is possible with the use of conventional organic synthesis.

35 Furthermore, the synthesis of fatty acid esters with the use of such an enzyme in a cell free method, such as in a bioreactor can be more efficient and less restricted since the method is only limited to the conditions by which the enzyme is active, whereas in a fermentation method the limitations is set by the conditions for the maintenance of the microorganisms. In such a fermentation system the fatty acid

ester products to be synthesised is limited to the building components, such as acyl donors and acyl acceptors present within the cell, whereas in a cell free system the limitation is only set by the properties of the enzyme such as substrate specificity. In a cell free system it is easy to calculate the amounts of the building components which are necessary to add to obtain an optimised enzyme catalysed method in which most of the building components ends up in the desired products such as fatty acid esters. Moreover, the use of lipases in a method for the synthesis of fatty acid esters is limited to water free conditions whereas membrane independent acyltransferases catalyses the acyl transfers in water containing systems.

Furthermore, use of a membrane independent acyltransferase as compared to a microorganism for the synthesis of for example lysophospholipids and/or fatty acid esters reduces the need of removing the microorganism after the synthesis is finalised

By the use of the new improved enzyme according to the invention it is possible to produce structured lipids without the need of organic solvents, which would be both environmentally favourable, healthier and eliminates one or more purification steps after the production of the structured lipids. Additionally, it may be easier to get an approval by the authorities for such a product, manufactured in a process without the use of organic solvents.

DESCRIPTION OF THE DRAWINGS

The invention is illustrated with reference to the drawings in which

Fig 1 shows Western blot analysis using protein extract of the cell free supernatant from growth of *Pichia pastoris* KM71H transformed with the pATWAX construct.

Fig 2 shows the synthesis of triacylglycerol catalysed by the membrane independent acyltransferase (ATWAX), as visualized by autoradiography of lipid products separated on TLC.

Fig 3. shows the time course of the wax esters synthesises from added soy lecithin and 13c-docosenoyl-alcohol (▣) or ricinoleoyl-alcohol (★) in cell free medium of *Pichia pastoris* cultures expressing the membrane independent acyltransferase HisATWAX as described in EXAMPLE 5.

Fig 4. shows the dependence of the ratio of the lecithin and ricinoleoyl-alcohol substrates (panel A) and increased substrate concentration with a fixed ratio of the substrates (panel B) on the wax ester synthesis were determined as described in EXAMPLE 5.

Fig 5. shows acyl group composition of soy lecithin (filled bars) and wax esters (open bars) produced from soy lecithin and ricinoleoyl alcohol as described in EXAMPLE 5. Abbreviations used; palmitoyl (16:0), palmitoleoyl (16:1), stearoyl (18:0), oleoyl (18:1), linoleoyl (18:2), linolenoyl (18:3).

5 Fig 6. shows microsomes prepared from wild type *Saccharomyces cerevisiae* cells, overexpressing the yeast PDAT gene *LRO1*, catalyses synthesis of triacylglycerols but not wax esters. Acyltransferase activities were analysed in the presence of the substrate *sn*1-oleoyl – *sn*2 – [^{14}C]oleoyl-phosphatidylcholine (lane 1) together with either ricinoleoyl alcohol (Ric-OH, lane 2) or 13c-docosenol (22:1-

10 OH, lane 3) as described in EXAMPLE 6.

Fig 7. shows the alignment of *Saccharomyces cerevisiae* phospholipid: diacylglycerol acyltransferase (ScPDAT) amino acid sequence, encoded from the *LRO1* gene, with the *Schizosaccharomyces pombe* SpPDAT, *Arabidopsis* At6704, At1254, At3027, At4557 and the *Crepis alpina* Cp6704 and Cp1254 deduced amino

15 acid sequences.

Fig 8. shows part of the *Saccharomyces cerevisiae* phospholipid: diacylglycerol acyltransferase (ScPDAT) amino acid sequence, encoded from the *LRO1* gene, aligned with amino acid sequences translated from the AnPDAT and AfPDAT nucleic acid sequences.

20 Fig 9. shows the synthesis of triacylglycerol catalysed by the membrane independent acyltransferase (HisATWAX-P6), as visualized by autoradiography of lipid products separated on TLC.

DETAILED DESCRIPTION OF THE INVENTION

25

Definitions

In the context of the present application and invention the following definitions apply:

The term "nucleotide sequence" is intended to mean a sequence of two or

30 more nucleotides. The nucleotides may be of genomic, cDNA, RNA, semi synthetic or synthetic origin or a mixture thereof. The term includes single and double stranded forms of DNA or RNA.

The term "deleted and/or substituted" is intended to mean that one or more amino acid residue(s) is/are removed (deleted) from the polypeptide and/or changed

35 (substituted) into another amino acid(s).

The term "nucleotide sequence molecule" is intended to indicate a consecutive stretch of three or more regions of nucleotide sequences. The nucleotide sequence molecule comprises a promoter region, a nucleotide sequence and a non-translated region. The nucleotide sequence or nucleotide sequence molecule may be

of genomic, cDNA, RNA, semi-synthetic or synthetic origin, or a combination thereof. The nucleotide sequence molecule is designed to express a nucleotide sequence located within the nucleotide sequence molecule when the nucleotide sequence molecule integrated into the genome or within a microorganism.

5 The term "promoter region" is intended to mean one or more nucleotide sequences involved in the expression of a nucleotide sequence, e.g. promoter nucleotide sequences, as well as nucleotide sequences involved in regulation and/or enhancement of the expression of the structural gene. A promoter region comprises a promoter nucleotide sequence involved in the expression of a nucleotide
10 sequence, and normally other functions such as enhancer elements and/or signal peptides. The promoter region may be selected from a plant, virus and bacteria or it may be of semi-synthetic or synthetic origin or a mixture thereof as long as it functions in a microorganism. Example of a promoter region is the methanol oxidase promoter, which can be used for the expression of polypeptides in *Pichia*
15 *pastoris*.

The term "a non-translated region " also called termination region is intended to mean a region of nucleotide sequences, which typically cause the termination of transcription and the polyadenylation of the 3' region of the RNA sequence. The non-translated region may be of native or synthetic origin as long as it functions in a
20 microorganism according to the definition above.

The term "operably linked" is intended to mean the covalent joining of two or more nucleotide sequences by means of enzymatic ligation, in a configuration which enables the normal functions of the sequences ligated to each other. For example a promoter region is operably linked to a signal peptide region and/or a
25 coding nucleotide sequence encoding a polypeptide to direct and/or enable transcription of the coding nucleotide sequence. Another example is a coding nucleotide sequence operably linked to a 3' non-translated region for termination of transcription of the nucleotide sequence. Generally, "operably linked" means that the nucleotide sequences being linked are continuously and in reading frame.
30 Linking is normally accomplished by ligation at convenient restriction sites. If such sites do not exist, synthetic adaptors or the like are used in conjunction with standard recombinant DNA techniques well known for a person skilled in the art.

The term "acyltransferase" is intended to mean a polypeptide, which have the ability to catalyse the transfer of an acyl group from one molecule to another (*i.e.*
35 interesterification). This transfer involves the breakage of an ester or a thioester bound of the donor molecule and the formation of an ester or thioester bound between the transferred acyl group and the acceptor molecule. Hence, in principal any molecule with an ester/thioester-linked acylgroup can act as a donor molecule and a molecule with at least one hydroxy or a thiol group could act as an acceptor

molecule. Commonly occurring donor molecules are acyl-CoA or lipids such as phospholipids and the acyltransferases are in nature known to catalyse *e.g.*, with diacylglycerols, sterols and alcohols as acceptor molecules, the final step in the synthesis of the storage compounds triacylglycerols (TAG), steryl esters and wax
5 esters, respectively.

The term "lipid dependent acyltransferase" is intended to mean an acyltransferase as described above restricted to utilising lipids such as phospholipids, glycolipids, triacylglycerols or other acyl-lipids that could serve as the acyl donor in the acyltransfer reaction. The lecithin: cholesterol acyltransferase
10 (LCAT) (Jonas A., 2000, Biochem. Biophys.Acta 1529: 245-256) and the bacterial glycerophospholipid: cholesterol acyltransferase (GCAT) (Brumlik and Buckley, 1996, J. Bacteriol. 178: 2060-2064) are the only known lipid dependent acyl transferase that has been shown to be functionally active as soluble proteins. All other known lipid dependent acyltransferases are polypeptides with one or several
15 membrane spanning regions and is exemplified by the phospholipid: diacylglycerol acyltransferase (PDAT) and its homologues. It should also be noted that the LCAT enzyme is dependent on an apolipoprotein for functionality. The bacterial GCAT does not show any strong sequence homologies to neither the LCAT nor the PDAT enzymes or to any other known acyltransferases.

20 The term "membrane independent acyltransferase" are intended to mean an acyltransferase, which is functionally active without being via a membrane-spanning region integrated into a membrane. The "membrane independent acyltransferase" is also active in a water- based environment.

The term "enzymatic conditions" are intended to mean that any necessary
25 conditions available in an environment, which will permit the enzyme to function.

The term "membrane spanning region" is intended to mean part of a polypeptide which anchor the polypeptide into a membrane and is hydrophobic, i.e., the membrane spanning region, such as amino acid residue number 80- 96 of the polypeptide shown in SEQ ID NO:1 in the patent application WO 00/60095, as
30 predicted by a hydrophobic plot (Kyte, & Dolittle).

The term "stringent conditions" is intended to mean hybridisation and washing conditions which permits the hybridisation between related nucleotide sequences to be permitted during the hybridisation and remain hybridised during the washing, such as an overnight hybridisation at 42°C in a solution comprising 50%
35 formamide, 5 x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5xDenhardt's solution, 10% dextran sulphate and 20 mg/ml denatured sheared salmon sperm DNA followed by washing the hybridisation membrane or support in 0.1 X SSC at approximately 65 °C.

The term "acyl donors " are intended to mean phospholipids,

triacylglycerols or other molecules containing at least one esterified acyl group that can be donated to an acyl acceptor in the production of fatty acid esters and/or fatty acid thioesters.

The term "acyl acceptors" are intended to mean molecules with at least one hydroxy or thiol group, to which acyl groups derived from the acyl donors can be esterified in the formation of an fatty acid esters or thioesters.

The term "fatty acid esters" are intended to mean fatty acid esters produced by a membrane independent acyltransferase catalysing the formation of ester bounds as described herein from the above mentioned acyl donor and acyl acceptors. Examples of fatty acid esters are acyl-lipids such as triacylglycerols, diacylglycerols, monoacylglycerols, phospholipids, glycolipids, lysolipids etc; waxesters (*i.e.* fatty acids esterified with long chain alcohols); acylated carbohydrates; acylated amino acids; or any other molecules with at least one acyl group esterified to a hydroxyl group.

The term "stabiliser" is intended to mean any kind of stabilising agent used by persons skilled in the art in order to increase the stability and shelf life time of enzymes.

Nucleotide sequences, nucleotide sequence molecules or vectors of the invention

The invention relates to one or more nucleotide sequence(s), derived from a nucleotide sequence encoding an acyltransferase polypeptide comprising at least one membrane-spanning region, encoding an improved active membrane independent acyltransferase polypeptide in which at least one amino acid residue of the membrane-spanning region has been deleted and/or substituted as compared to the original acyltransferase polypeptide, wherein the encoded active membrane independent acyltransferase polypeptide can produce lysolipids and fatty acid esters and/or fatty acid thioesters such as lysophosphospholipid, lysolecithin, triacylglycerols, diacylglycerols, monoacylglycerols, phospholipids, glycolipids, waxesters, acylated carbohydrates and acylated amino acids. By deletion and/or substitution of one or more amino acid residues the encoded polypeptide looses the ability to become integrated into a membrane and remains membrane independent as compared to the original polypeptide . The numbers and/or the location of the amino acid residue(s) to be deleted and/or substituted is/are not critical as long as the polypeptide by the deletion and/or substitution become membrane independent. Part of the membrane-spanning region may be present as long as it does not integrate or attach the polypeptide to a membrane. The polypeptide encoding the membrane independent acyltransferase named ATWAX is a membrane independent acyltransferase which may be encoded by a nucleotide sequence, originally encoding an integral membrane protein with one or several membrane spanning

regions wherein one or several of the membrane spanning regions has/have been deleted and/or substituted. The nucleotide sequence may also be synthetic or semi synthetic as long as it has the function of a membrane independent acyltransferase which may be used in the formation of fatty acid esters, like acyl-lipids such as triacylglycerols, diacylglycerols, monoacylglycerols, phospholipids, glycolipids, lysolipids etc; waxesters (*i.e.* fatty acids esterified with long chain alcohols); acylated carbohydrates; acylated amino acids; or any other molecules with at least one acyl group esterified to a hydroxyl group. The nucleotide sequence encoding the acyltransferase may be derived from a nucleotide sequence encoding a lipid dependent acyltransferase polypeptide, such as a nucleotide sequence encoding an lipid dependent acyltransferase polypeptide catalysing an acyl transfer reaction in which acylphospholipids acts as acyl donors, for example a nucleotide sequence encoding a phospholipid: diacylglycerol acyltransferase.

Such nucleotide sequences may be obtained from different kind of species such as bacteria, yeasts, fungi, plants, insects or mammalians. Examples are *Arabidopsis thaliana, Crepis palaestina, Euphorbia lagascae, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Aspergillus* strains, *e.g. A. niger, A. nidulans, A. fumigatus, A. sojae, Pichia* strains, such as *P. Pastoris* or *P. methanolica Mucor* strains, *e.g. M. circinelloides, Hansenula,* such as *H. Polymorpha* and *Trichoderma, Klyveromyces,* or *Yarrowia.* Examples on nucleotide sequences are shown in SEQ ID NO:1, 4, 8, 10, 12, 14, 15, 16, 18 or 20.

According to one embodiment the invention relates to a nucleotide sequence, wherein from 1 to 291 nucleotide sequence residue(s) has/have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:1. The number(s) of nucleotide sequence residues to be deleted is/are chosen in such a way that the open reading frame of the nucleotide sequence encoding the membrane independent acyltransferase polypeptide is not disturbed and the membrane spanning region corresponding to nucleotide sequence 238 to 288 is deleted and/or substituted. One example is the nucleotide sequence shown in SEQ ID NO:2, where 290 nucleotide sequence residues have been deleted and/or substituted resulting in the membrane independent acyltransferase polypeptide shown in SEQ ID NO:3.

According to another embodiment the invention relates to a nucleotide sequence, wherein from 1 to 219 nucleotide sequence residue(s) of the 5'-end has/have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO: 4, 1-87 nucleotide sequence residue(s) of SEQ ID NO:8 and SEQ ID NO:10 and 1-190 nucleotide sequence residue(s) of SEQ ID NO:12.

According to another embodiment the invention relates to a nucleotide sequence, wherein at least the nucleotide sequence residues 142 to 210 have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:4,

19-87 nucleotide sequence residues of SEQ ID NO 8 and SEQ ID NO:10 and 130-190 nucleotide sequence residues of SEQ ID NO:12.

According to another embodiment the invention relates to a nucleotide sequence, wherein from 1 to 228 nucleotide sequence residue(s) of the 5'-end has/have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO: 16, 1-219 nucleotide sequence residue(s) of SEQ ID NO:18 and 1-261 nucleotide sequence residue(s) of SEQ ID NO:20.

According to another embodiment the invention relates to a nucleotide sequence, wherein at least the nucleotide sequence residues 169 to 228 have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:16, 151-219 nucleotide sequence residue(s) of SEQ ID NO:18 and 193-261nucleotide sequence residue(s) of SEQ ID NO:20.

The number(s) of nucleotide sequence residues to be deleted is/are chosen in such a way that the open reading frame of the nucleotide sequence encoding the membrane independent acyltransferase polypeptide is not disturbed and the membrane spanning region are removed/deleted.

According to one embodiment of the invention the nucleotide sequence encoding the membrane independent acyltransferase polypeptide may hybridise under stringent conditions to a nucleotide sequence as shown in SEQ ID NO:1, 2, 4, 6, 8, 10, 12, 14, 15, 16, 18 or 20. Furthermore the nucleotide sequence as shown in SEQ ID NO: 1, 2, 4, 6, 8, 10, 12, 14, 15, 16, 18 or 20 may be different as compared to another nucleotide sequence due to the degeneracy of the genetic code.

Additionally the nucleotide sequence encoding the membrane independent acyltransferase polypeptide may at least show 75 %, 80%, 85%, 90% or 95% homology to the amino acid sequence(s) shown in SEQ ID NO:3, 7, 9, 11, 13, 17, 19, 21or a homologue thereof.

Furthermore, the nucleotide sequence shown in SEQ ID NO 2, encoding the membrane independent acyltransferase polypeptide shown in SEQ ID NO 3, may be modified by removing (deleting) nucleotides, encoding one or several amino acid residues in the N-terminal part corresponding to the first 71 amino acid residues of the polypeptide shown in SEQ ID NO 3, with maintained acyltransferase activity. Furthermore one or more amino acid residues may be substituted as long as the acyltransferase activity remains. Methods, which are suitable for the removal (deletion) of a specific nucleic acid sequence are well known for a person skilled in the art and includes methods such as PCR.

Moreover, the amino acid residues S229, D472, and H523 shown in SEQ ID NO 3 are essential for activity as described in the examples and is here suggested to be part of the a catalytic triad in the active site of the enzyme.

Additionally, the invention relates to an oligonucleotide, which specifically hybridise under stringent conditions to the nucleotide sequence(s) and/or the nucleic acid molecule(s) described herein. The oligonucleotide may be used for the detection of the nucleotide sequence and/or the nucleotide sequence, such as the
5 presence of the nucleotide sequence within a host cell.

According to another embodiment the invention relates to a nucleotide sequence molecule, which comprises at least one promoter region which functions in a host. The promoter region is operably linked to at least one nucleotide sequence as described above, which is operably linked to at least one non-translated region
10 which functions in a host. Furthermore, a signal peptide may be present between the promoter region and the nucleotide sequence as described above.

The nucleotide sequence molecule may be present in a vector, such as an expression vector, which may be used for the production of the polypeptide, which has acyltransferase activity. The vector is typically derived from plasmid or viral
15 DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Any kind of vector may be used as long as it functions in a host cell which is capable of performing glycosylation of the polypeptide, such as vectors which functions in yeast. Useful expression vectors for yeast cells include the 2μ plasmid and
20 derivatives thereof, the POT1 vector (US 4,931,373), the pJSO37 vector described in Okkels, Ann. New York Acad. Sci. 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen).

Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide to be amplified in copy number. Such
25 amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrafolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS")
30 amplification (see, e.g., US 5,122,464 and EP 338,841).

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2μ replication genes REP 1-3 and start of replication.
35 The vector may also comprise a selectable marker, e.g., a gene the product of which complements a toxin related deficiency in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g., ampicillin, kanamycin, tetracyclin, chloramphenicol,

neomycin, hygromycin, zeocin or methotrexate. For *Saccharomyces cerevisiae*, selectable markers include *ura3 and leu2*. For filamentous fungi, selectable markers include *amdS*, *pyrG*, *arcB*, *niaD* and *sC*.

The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of the polypeptide of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader sequence, signal peptide, polyadenylation sequence, propeptide sequence, promoter (inducible or constitutive), enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter. Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast α-mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the inducible GAL promoter. Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* α-amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidulans* acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator.

The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide to be expressed (whether it is an intracellular or extra cellular polypeptide) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. For use in yeast cells suitable signal peptides have been found to be the α-factor signal peptide from *S. cereviciae* (cf. US 4,870,008), a modified carboxypeptidase signal peptide (cf. L.A. Valls *et al.*, Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani *et al.*, Yeast 6, 1990, pp. 127-137), and the synthetic leader sequence TA57 (WO98/32867).

Furthermore the invention relates to an oligonucleotide, which hybridises under stringent conditions (as defined above) to a nucleotide sequence and/or a nucleotide sequence molecule as described above.

<u>Host cells, method and polypeptide of the invention</u>

Any suitable host cell may be used for the maintenance and production of the vector of the invention as long as the host is capable of producing a glycosylated product. The host cell may be a eukaryotic cell, for example fungi, yeast, insects and mammalian cells. A eukaryotic system may provide significant advantages
5 compared to the use of a prokaryotic system, for the production of certain polypeptides encoded by nucleotide sequence molecules and/or vectors present within the host cell or integrated into the genome of the host cell. For example, yeast can generally be grown to higher cell densities than bacteria and may be capable of glycosylating expressed polypeptides, where such glycosylation is
10 important for a proper folding of the polypeptide and/or catalytic activity of the polypeptide.

The host cell may be a host cell belonging to a GMP (Good Manufacturing Practice) certified cell-line. Examples of suitable filamentous fungal host cells include strains of *Fusarium, Trichoderma, Aspergillus,* e.g. *A. oryzae, A. niger, A.*
15 *sojae* or *A. nidulans, Mucor, e.g. M. circinelloides.* Examples of suitable yeast host cells include strains of *Saccharomyces,* e.g. *S. cerevisiae, Schizosaccharomyces, Klyveromyces, Pichia,* such as *P. Pastoris* or *P. methanolica, Hansenula,* such as *H. Polymorpha* or *Yarrowia.* Examples of *P. Pastoris* strains are X-33, KM71H, and GS115 which may be obtained from Invitrogen Inc. *Pichia pastoris* is a
20 methylotrophic yeast which can grow on methanol as a sole carbon and energy source (Ellis et al., 1985). *P. pastoris* is also amenable to efficient high cell density fermentation technology. Therefore is *Pichia pastoris* a suitable host for expression of heterologous protein in large quantity, with a methanol oxidase promoter based expression system (Cregg et al., 1987). Additional suitable donor cell lines are
25 known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland.

The vector is transferred (introduced) into the host cell using a suitable method dependent on which host cell has been selected. The introduction of the vector harbouring the nucleotide sequence molecule into fungal cells may be by a
30 method involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se.* Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and US 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156 and WO 96/00787. Yeast may be
35 transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology,* Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153: 163; Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920: and as disclosed

by Clontech Laboratories, Inc, Palo Alto, CA, USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit) or by using the Pichia Manual supplied by Invitrogen Inc. These methods are well known in the art and e.g., described by Ausbel *et al.* (eds.), 1996, Current Protocols in Molecular Biology,
5 John Wiley & Sons, New York, USA.

In the production methods (process) of the present invention, the cells are cultivated in a growth medium suitable for maintenance and/or production of the nucleotide sequence molecule and/or the vector using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-
10 scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable growth medium and under conditions allowing the vector, nucleotide sequence molecule or polypeptide to be expressed and/or isolated. The vector, nucleotide sequence molecule or the polypeptide may be used in the chemical or in the pharmaceutical
15 industry. The cultivation takes place in a suitable growth medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable growth media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). The cultivation of *Pichia pastoris* is performed using the
20 method described in EXAMPLE 2 or any other suitable method. After cultivation, the polypeptide is recovered from the culture medium, the cells or after separating the cells from the culture medium. The recovered polypeptide encodes an active membrane independent acyltransferase without the ability to become integrated into a membrane, i.e., one ore more of the amino acid residue(s) present in the
25 membrane spanning region has/have been deleted and/or substituted. Examples of methods are those mentioned in Maniatis et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbour Press) (1989) and Qiagen Inc.

According to another embodiment the polypeptide is an acyltransferase active at a pH ranging from about 4 to about 10, and stable at a temperature below
30 about 60 °C. The enzymatic activity of the polypeptide can be measured using the assay method described in EXAMPLE 4 or EXAMPLE 5.

The polypeptide may furthermore be immobilised to a carrier. Suitable carriers and methods for the immobilisation of the polypeptide to the carrier are well known for a person skilled in the art (Tisher, W., & Kasche, V., 1999, Trends
35 Biotechnol. 17(8): 326-335).

According to one embodiment of the invention the polypeptide named ATWAX and described above may be lyophilised and/or freeze-dried. Lyophilisation and/or freeze-drying may be performed using conventional techniques known for a person skilled in the art.

A further embodiment relates to the use of a nucleotide sequence and/or a nucleotide sequence molecule and/or a vector and/or a host cell and/or the methods and/or the polypeptide of the invention. The polypeptide may be used for the production of fatty acid esters. Examples of fatty acid esters are acyllipids such as triacylglycerols, diacylglycerols, monoacylglycerols, phospholipids, glycolipids, lysolipids; waxesters; acylated carbohydrates; acylated amino acids; or any other molecules with at least one acyl group esterified to a hydroxyl group are fatty acid esters.

Examples of products which may be produced by the method are fatty acid esters used in cosmetics, foods, food additives, dairy products, confectionary, flavours, bakery, pharmaceuticals, candles, soaps, detergents, laundries, polymers, coatings, plasticizer-, drying oils, lubricants, varnishes, linoleum, printing, inks, textile dyes and surfactants.

Examples of what the invented polypeptide may be used for are listed below.

Production of structured lipids

The invented polypeptide(s) are suitable to be used in the production of structured lipids without the need of organic solvents, which would be both environmentally favourable, healthier and eliminates one or more purification steps after the production of the structured lipids. Additionally, it may be easier to get an approval by the authorities for such a product, manufactured in a process without the use of organic solvents. The positional distribution of acyl groups differing in length and degree of saturation in the triacylglycerol molecule is known to be important regarding nutritional and health aspects partly due to differences in digestibility and absorbability. As an example most triacylglycerols of vegetable origin are highly unsaturated at the 2- position, mainly oleic and linoleic acid. However, in human milk fat the saturated fatty acid palmitic acid is highly enriched at the 2-position and it is known that such type of fat is more easily absorbed and utilized by infants. Structured triacylglycerols mimicking the properties of triacylglycerols in the human milk fat can be manufactured in a process in which ATWAX catalyses the transfer of acyl groups from the sn2 position of lecithin to monoacylglycerol with palmitic acid at the sn2 position (2- palmitoyl glycerol), yielding triacylglycerols enriched with unsaturated fatty acids such as oleic and linoleic acid in the 1-, 3- positions and palmitic acid in the 2-position. The source of the 2- palmitoyl glycerol for use in this process may be obtained by 1, 3 specific lipase hydrolyses of palm oil enriched in triacylglycerols with palmitic acid at the 2-position.

In another application structured lipids are used as fat replacers in low calorie foods. Acylglycerols with an acetyl group at the 2- position are used as fat replacers

in low calorie foods such as dairy products, bakery, cereals, pasta, cheese, tofu, chocolate, chocolate confections, margarine, salted snacks, sour cream, spreads etc. This diet fat can be manufactured in a process in which ATWAX catalyse the transfer of acyl groups from the sn2 position of lecithin to 2- acetyl glycerol. In a
5 similar manner, structured diacylglycerols can be produced by an acyltransfer of fatty acids from an acyldonor such as lecithin to glycerol as the acceptor molecule. The major product in such a process is 1,3-diacylglycerol (i.e. diacylglycerol with acyl groups at the sn1 and sn2 position), since the ATWAX enzyme has preferences for the acylation of the sn1 and sn3 positions of the glycerol molecule.

10

Production of fat-soluble molecules.

The invented polypeptide(s) are suitable to be used to render molecules more hydrophobic by coupling fatty acids via an acylation reaction to molecules that otherwise are badly soluble in hydrophobic solvents such as fats and oils. Example
15 of such modification, is the acylation of water soluble flavours and vitamins which makes these fatty acid esters of flavours and vitamins more fat soluble and hence more suitable for certain applications such as food, cosmetic, and pharma applications. As an example fatty acid esters of vitamins such as vitamin E (tocopherol) are used in skin-care products since the vitamins are more readily
20 adsorbed into the skin. In certain food applications it is desirable to make water-soluble flavours, vitamins or other additives are more easily mixed into fatty foodstuffs. In a process involving ATWAX the manufacturing of fatty acid esters by the acylation of hydrophilic molecules possessing a hydroxyl group can be performed by an acyltransfer catalysed by ATWAX. The acyl donor molecule in
25 this reaction can be lecithin or phospholipids or any other suitable acyl-lipids.

Removal of undesirable fat.

The invented polypeptide(s) are suitable to be used enables the removal of one or more fatty acids from a molecule by the use of ATWAX, such as by
30 transferring one or more fatty acids from a molecule to an acceptor molecule such as monoacylglycerol, diacylglycerol. Phospholipids present within milk and dairy products are examples of molecules from which one fatty acid may be removed are. The major phospholipids present in milks from mammals are phosphatidylcholine, phosphatidylethanolamine and sphingolipids, each comprising about 30% of the
35 total phospholipids present. These phospholipids are part of the milk fat globule membrane fraction, which constitutes a minor part of the whole milk lipids. Apart from the phospholipids this membrane lipid fraction also contains TAG, diacylglycerol and monoacylglycerol.

It is desirable to remove fatty acids, especially unsaturated fatty acids such as

oleic, linoleic, and linolenic acid from milk prior to use of the milk in products such as low fat or non fat products. During storage the unsaturated fatty acids that are mainly present on the sn2-position of the phospholipids, becomes oxidised and thus the milk product becomes rancid with a bad smell and taste (off-flavour). Today,
5 there is no suitable method for removal of these undesirable fatty acids from milk. By the addition of ATWAX to milk the fatty acid on the sn2 position of the phospholipid is transacylated to the acceptor molecule such as monoacylglycerol and/or diacylglycerol by which triacylglycerol is produced. This formed TAG will be removed together with the main TAG, in the production of low fat or non-fat
10 product such as dry milk powder, cheese, yoghurt and other dairy products. Thereby the off-flavouring is reduced and/or eliminated and the shelf life time of the products could be increased.

Furthermore, in the transfer of fatty acids in milk from the phospholipids to acceptor molecules such as monoacylglycerols or diacylglycerols, the phospholipids
15 will be converted to lysophospholipids. With an increased fraction of lysophospholipids, the membrane lipid fraction is more easily disintegrated and the encapsulated TAG is released. This released fraction of TAG as well as the TAG that is formed in the transfer of fatty acids from lecithin to monoacylglycerols and/ or diacylglycerol will be removed together with the main TAG fraction. Thereby a
20 process, in production of low-fat or non-fat milk products, involving the use of ATWAX can more efficiently reduce the fat content in such milk products.

Another field of application is to use ATWAX to remove phospholipids e.g. lecithin. In the refining of vegetable oils, the removal of the lecithin fraction, i.e. degumming is an important process in the production of high quality oils. In a
25 refining process involving the use of ATWAX, lecithin present in the oil can be converted into lysolecithin, which will be removed from the oil into the water phase. The fatty acid removed from the lecithin in this process will be transferred to an acceptor molecule present in the oil such as diacylglycerol by which triacylglycerol is formed.

30

Modification of lipids presents in animal and plant raw material.

In the field of baking, bread improvers such as emulsifiers based on lipids are commonly used. However, these emulsifiers are known to give off-flavour and also caking and lumping problems, especially in hot and humid climates. In flour, such
35 as wheat flour, polar lipids mainly lecithin and galactolipid (e.g. digalctosyldiacylglycerol) are present. In a baking process in which ATWAX is added, the lecithin and the galactolipid deriving from the flour, can enzymatically be converted into the corresponding lysolipids. This conversion of the polar lipids into lysolipids are known to give a similar stabilising effect of the dough as what is

achieved with the today commonly added emulsifiers such as diacetyl tartaric acid esters of monoacylglycerols. Therefore the ATWAX enzyme can totally or partly replace the use of emulsifiers in the baking process and thus reduce the problems with off-flavour and the tendency of lump formation.

5 In a similar manner the conversion of lecithin or phospholipids present in "raw materials" such as milk, flour, eggs, soy protein, cocoa, or any other animal or plant materials into lysolecithin or lysophospholipids can be executed in a process involving ATWAX. In such as process important properties of the raw material are modulated, such as amphiphilic nature, texture, melting point, viscosity, flavour, 10 emulsification, foaming, and wetting, to be suited for the production of a certain complex foodstuffs. Thereby, the need for food additives such as emulsifiers, wetting agents, dough strengtheners, and film formers are reduced.

Kit of the invention

15 A kit comprising the polypeptide which has the enzymatic activity of a (membrane independent) acyltransferase and the membrane spanning region removed or a fragment thereof or a kit in which the polypeptide has been immobilised on a carrier. . The polypeptide may be provided in the kit as lyophilised or freeze dried. The kit may also comprise components, which are 20 essential for the stability and activity of the polypeptide, such as a stabiliser. The kit may furthermore comprise a manual with instructions for the use of the polypeptide.

The following examples are intended to illustrate but not to limit the invention in any manner, shape, or form, either explicitly or implicitly.

25 EXAMPLES

EXAMPLE 1

Amplification of nucleotide sequences, homologues to the *Saccharomyces* 30 *cerevisiae* gene *LRO1*, for expression in *Pichia pastoris*.

Nucleotide sequences were amplified from a plasmid template (pBluescript, Stratagene Inc.) containing the intact yeast gene *LRO1*, encoding a phospholipid: diacylglycerol acyltransferase (PDAT) with one membrane-spanning region, (described in Dahlqvist et al., 2000, PNAS 97:6487-6492) by thermo stable Pfu 35 Turbo Polymerase (Stratagene Inc.). A set of primers were designed for the amplification of three different nucleotide sequences A, B, and C, identical in nucleotide sequence to the part of the *LRO1* gene encoding the amino acid residues 98 to 661, 170 to 661 and 190 to 661 respectively. The forward (5' end) primers used for this PCR reaction were for the sequence A, B, and C;

5'CCATGGGAATGAATTCATGGCTTATCATGTTCATAATAGCGATAGC3',
5'CCATGGGAATGAATTCCGAGGCCAAACATCCTGTTGTAATG3', and
5'CCATGGGAATGAATTCGGAGTTATTGGAGACGATGAGTGCGATAGT3',
respectively.

5 The oligonucleotide sequence,
5'GCCTCCTTGGGCGGCCGCTCACATTGGGAGGGCATCTGAGAAAC3'
was used as the reverse (3' end) primer in all the three PCR amplifications. The three amplified nucleotide sequences all lack the sequence region, present in the *LRO1*, which encodes a transmembrane region. The amplified nucleotide sequence
10 A is shown in SEQ ID NO 2.

Additionally one nucleotide sequence was amplified resulting in the nucleotide sequence D with nucleotides encoding 6-residue histidine at the N-terminus in frame with the region encoding the amino acid residues 98 to 661 of the
15 yeast PDAT. This was achieved by first sub cloning the amplified nucleotide sequence A above into the NheI and XhoI sites of the plasmid pET28a(+) and then using the following oligonucleotide primers for PCR amplification;
5'CCATGGGAATGAATTCATGGGCAGC AGCAGCCATCATCAT3' and
5'GCCTCCTTGGGCGGCCGCTCACATTGGGAGG GCATCTGAGAAAC3'.
20 The amplified PCR products A, B, C, and D above, were purified, digested by EcoRI and NotI, and subcloned between the EcoRI and NotI sites of the Pichia expression vector PpicZ□A in frame with the sequence encoding the □factor signalpeptide present in the expression vector. The resultant Pichia expression vectors are named pATWAX, p72ATWAX, p92ATWAX, and pHISATWAX, with
25 inserts encoding the polypeptides ATWAX, 72ATWAX, 92ATWAX, and HISATWAX respectively. These vectors were linearized using unique SacI restriction site for transformation in *Pichia pastoris* host strain.
Site directed mutagenesis of the ATWAX polypeptide sequence described in SEQ ID NO: 2 were performed in order to identify the catalytic triad. The PCR
30 based mutagenesis were performed using mega-primer method (Ling, M. M., & Robinson, B. H., 1997, 254(2): 157-178) for the construction of three nucleotide sequences encoding ATWAX-S229A, ATWAX-D472N and ATWAX-H523A with the single residue mutant S229A, D472N and H523A, respectively.

EXAMPLE 2

35 Transformation in Pichia and growth for expression.

Competent *Pichia pastoris* cells were prepared according to the procedure mentioned in the EasySelect Manual supplied by Invitrogen Inc. Electroporation, as

described in the EasySelect Manual, was used to transform the linearized expression vector, pATWAX, p72ATWAX, p92ATWAX or pHISATWAX described in EXAMPLE 1 above, into the *Pichia pastoris* host strain X-33 or KM71H. The procedure of Zeocin selection was used to select transformants, which were plated
5 on YPD medium containing Zeocin. For the expression of the transformed genes, cells were initially cultured to a final O.D. of 3-5 in BMGY medium supplemented with 1% (v/v) glycerol, after which cells were subsequently washed with either sterile water or YPD medium. The washed cells were then suspended in BMMY medium supplemented with 0.5% (v/v) methanol for induction of the transgene and
10 further cultured for 3-4 days in a volume corresponding to 0.5-0.2 of the original volume. Methanol (20%, v/v) was added to a final concentration of 0.5% (v/v) every 24 hours. Cell-free medium was collected by centrifugation and was used for western blot analyses and enzyme activity studies.

15

EXAMPLE 3

Western blot analysis of cell free medium of *Pichia pastoris* KM71H transformed with pATWAX.

In order to determine the presence of ATWAX in the cell free culture
20 medium, *P. pastoris* KM71H transformed with pATWAX were cultured as described in EXAMPLE 2. Aliquots of cell free culture medium were withdrawn at different time points following induction and subjected to Western blot analysis using anti yeast-PDAT polyclonal antibody. The antibody was raised in a rabbit by the injection of partially purified ATWAX. The ATWAX used for this purpose was
25 produced in *Echerichia coli.*

The western blot based on immunodetection system as presented in figure 1 clearly show the presence of a polypeptide, present in the cell free medium with a molecular weight of approximately 82 kDa, that cross-reacts with the anti-yeast PDAT. By comparing the results obtained on the western blot analyses obtained
30 with the cell free medium from 58, 82 and 112 hours of induction (*i.e.* Fig. 1 lane 1, 2, and 3, respectively) it is concluded that the secreted ATWAX is continuously secreted and accumulated in the cell free medium up to at least 112 hours of induction without being degraded. This is further supported by the lack of additional band of lower molecular weight that could be referred to as degradation
35 products. Cell free medium of the untransformed Pichia strain did not crossreact with the ATWAX antibody (lane5). These data also indicates that the ATWAX present in the cell free medium is glycosylated and that the glycosylation

contributes to about 17 kDa of the molecular weight, since a non-glycosylated ATWAX should have a weight of 65 kDa as calculated from its amino acid composition.

EXAMPLE 4

5 Detection of ATWAX enzyme activity in the culture supernatants.

To determine the enzyme activity in the cell free culture medium the cell free supernatant was assayed for enzyme activity as follows. The cell free supernatant of the induced cultures described in EXAMPLE 2, from 116 hours of growth of *Pichia pastoris* KM71H transformed with the pATWAX construct encoding ATWAX, the
10 polypeptide described in SEQ ID NO: 2 (Fig 2, lane 2) and the congenic wt strain (Fig 2, lane 1) were assayed for acyl transferase activity. Lipid substrate, *sn1*-palmitoyl *sn2*-[^{14}C]linoleoyl- phosphatidylethanolamine (5 nmol; 5000 dpm/ nmol) and dioleoylglycerol (2.5 nmol) dissolved in chloroform was aliquoted in 1.5 ml tubes and the chloroform was evaporated under a stream of N_2 (g). After addition of
15 20 ul 0.25 M potassium phosphate, pH 7.2, the mixture was violently agitated and 80 ul of cell free supernatant was added and incubated at 30°C for 90 min. Lipids were extracted from the reaction mixture into chloroform (Bligh, E.G. and Dyer, W.J (1959) Can. J. Biochem. Physiol. 37, 911-917) and separated by TLC on silica gel 60 plates (200 x 200 mm) in chloroform / methanol / acetic acid / water
20 (85:15:10:3.5) migrating 90 mm using an automatic developing chamber (Camag). The plate was dried and redeveloped in hexane / diethyl ether / acetic acid (80:20:1) with a solvent migration of 180 mm. The radioactive lipids were visualized and quantified on the plates by electronic autoradiography (Instant Imager; Packard). As a control, the enzyme activity in the cell free supernatant of the wild type host strain
25 culture was analysed. As shown in Figure 2, the majority of the [^{14}C]linoleoyl group translocated from phosphatidylethanolamine is associated with triacylglycerol after the incubation. This demonstrates that the truncated membrane independent form of yeast PDAT, referred to as a membrane independent acyltransferase that we have named ATWAX, is able of catalysing the formation of TAG by an acyltransfer from
30 phosphatidylethanolamine to diacylglycerol (DAG). Radiolabeled acylgroups can also be detected as unesterified fatty acids indicating the presence of a lipase activity. However, since a release of radiolabeled fatty acids also occur in the cell free supernatant of untransformed host strain it is not possible to conclude whether this lipase activity is associated with ATWAX.

35 The cell free supernatant of the induced cultures of *P. pastoris* KM71H transformed with the p72ATWAX or p92ATWAX constructs were by western blot

analyses shown to be expressed and secreted in to the culture medium. However the cell free medium containing these truncated polypeptides, lacking a stretch of 72 or 92 amino acids residues of the ATWAX N-terminus, respectively, did not catalyse the synthesis of TAG when analysed for enzyme activity according to method described above.

Furthermore, nucleotide sequences encoding the ATWAX-S229A, ATWAX-D472N and ATWAX-H523A mutant polypeptides were generated as described in EXAMPLE 1 and expressed in *Pichia pastoris* as described in EXAMPLE 2. The expression of these mutant polypeptides was verified by western blot analyses of aliquots of cell free medium from cultures expressing these polypeptides, respectively. However, all three mutant polypeptides were inactive when assayed for acyltransferase activity according to method described above. Hence, the amino acid residues S229, D472 and H523 are essential for the catalytic activity and are therefore here suggested to be part of a catalytic triad.

A membrane independent acyltransferase with an N-terminal stretch of six Histidine residues was produced by the expression of the construct HisATWAX in *Pichia pastoris* (described in EXAMPLE 1 and 2). This polypeptide was when analysed for acyltransferase activity as described above shown to be active with similar catalytic properties as the ATWAX.

EXAMPLE 5

<u>Production of waxesters</u>

Wax esters can be synthesised from soy lecithin and different alcohols by the catalyses of the membrane independent acyltransferases, ATWAX and HisATWAX. This acyltransferases was produced and secreted into the culture medium by expressing the construct pATWAX or pHisATWAX, respectively in *Pichia pastoris* (described in EXAMPLE 2). Aliquots of cell free medium was prepared and stored at –20 °C.

The ability to synthesise wax esters from soy lecithin and 13c-docosenoyl-alcohol ([illegible] in Fig 3) or ricinoleoyl-alcohol (★ in Fig 3) by the membrane independent His-tagged acyltransferase present in the cell free culture medium was investigated and the results are given in figure 3. The conditions for the synthesis were as follows; lecithin (2.5 mg), *sn1*-oleoyl-*sn2*-[^{14}C]oleoyl-phosphatidylcholine (5 nmol; 5000 dpm/ nmol) and 6 umol of 13c-docosenoyl-alcohol or ricinoleoyl-alcohol dissolved in chloroform was aliquoted in 12 ml glass tubes and the chloroform was evaporated under a stream of N_2 (g). To the dry lipid substrate 0.5 ml of cell free medium and 25 ul 1.0 M potassium phosphate, pH 7.2 were added.

The reaction mixture was sonicated in a water bath (Branson 2510) for 30 min and was further incubated at 37 °C to a final incubation time as indicated in figure 3. Lipids were extracted from the reaction mixture into chloroform (Bligh, E.G. and Dyer, W.J (1959) Can. J. Biochem. Physiol. 37, 911-917) and separated by TLC on silica gel 60 plates (200 x 200 mm) in hexane / diethyl ether / acetic acid (55:45:0.5) with a final solvent migration of about 180 mm. Wax esters products were verified through the methylation of the wax ester products excised from the TLC plate (using method described in Dahlqvist et al., 2000, PNAS 97: 6487-6492) followed by the separation of the methylation products on silica gel 60 plates in hexane / diethyl ether / acetic acid (55:45:0.5). Only two components were detected, methyl esters of fatty acids and free alcohols as identified by means of appropriate standards. The amounts of wax esters produced, from the added radiolabeled *sn1*-palmitoyl *sn2*- [^{14}C]linoleoyl- phosphatidylcholine and the non-labelled alcohols, were quantified on the plates by electronic autoradiography (Instant Imager; Packard) as percentage of radiolabel in wax esters of total added. As shown in figure 3 the ATWAX was catalysing the synthesis of wax esters of 13c-docosenoyl-alcohol (▦ in Fig 3) or ricinoleoyl-alcohol (★ in Fig 3) with similar efficiencies, which reached a plateau after 4 hours of incubation at which about 55 - 60 % of the radiolabeled acylgroups of the added phosphatidylcholine had formed a wax ester with the alcohol. Apart from the formation of the wax ester, lysophospholipids is also formed in this reaction.

The dependence of the ratio of the added lecithin and alcohol substrate on the conversion rate is presented in figure 4A. The conditions for the synthesis were as follows; lecithin (10 mg), *sn1*-oleoyl-*sn2*-[^{14}C]oleoyl-phosphatidylcholine (10 nmol; 5000 dpm/ nmol) and 3.7, 7.4, 11.1, or 18,7 mg of ricinoleoyl-alcohol, giving an alcohol to lecithin ratio as indicated in figure 4A, dissolved in chloroform was aliquoted in 12 ml glass tubes and the chloroform was evaporated under a stream of N_2 (g). To the dry lipid substrate 1.2 ml of cell free medium and 60 ul 1.0 M potassium phosphate, pH 7.2 was added. The reaction mixture was sonicated in a water bath (Branson 2510) for 30 min and was further incubated at 37 °C to a final incubation of 4 hours. Lipids were extracted and analysed as described above. These analyses show that at a weight ratio of the alcohol to lecithin of 0.4 (corresponding to an equimolar amounts of alcohol and lecithin added) 28 % of the radiolabeled fatty acids was converted into waxesters and by increasing the ratio 5-fold the wax ester synthesis was increased 2-fold.

By increased substrate concentration with constant lecithin to alcohol weight ratio, the conversion into waxesters is decreased as shown in figure 4B. Incubating lipid substrates as described above together with 1.2 ml of cell free medium at 37 °C for 20 hours performed these analyses. From the results presented in figure 4 A

and B it is evident that in order to optimise the yield of waxesters produced from lecithin and an alcohol the total substrate concentration and the substrate ratio are important factors to consider.

The major lipid component in the soy lecithin is phosphatidylcholine and 5 phosphatidylethanolamine constituting about 60 % of the acyl lipids present, other phospholipids present are phosphatidylinositol, phosphatidylglycerol and phosphatidic acid, which contributes up to approximately 25 %, the remaining lipids are neutral lipids, lysolipids, and glycolipids. The fatty acid composition of the total lipid content of the soy lecithin used in the present study was analysed and 10 are presented in figure 5 (filled bars). The major fatty acid component is linoleic acid (18:2) constituting 56 % of the fatty acids present, other unsaturated fatty acids are oleic acid (18:1) and linolenic (18:3), and the unsaturated fatty acids are palmitic (16:0) and stearic acid (18:0). In figure 5 data are also presented on the fatty acid composition of the wax ester (open bars) produced from the soy lecithin 15 and ricinoleoyl alcohol as described above. By comparing the fatty acid composition of the wax ester product with that of the lecithin substrate it is clear that the unsaturated fatty acids are preferentially converted into the waxesters whereas the saturated fatty acids are less efficiently used for wax ester synthesis. This can partly be explained by the fact that these unsaturated fatty acids are 20 preferentially esterified to the sn1 position of the lipids and that ATWAX is specific for the transfer of fatty acids from the sn-2 position.

The synthesis of different waxesters from soy lecithin was achieved by using different alcohols as the acyl acceptor and with conditions as follows; lecithin (2.5 mg), *sn1*-oleoyl-*sn2*- [^{14}C]oleoyl-phosphatidylcholine (10 nmol; 5000 dpm/ nmol) 25 and 6 umol of decanol, hexadecanol, 13c-docosanol, hexacosanol or ricinoleoyl-alcohol dissolved in chloroform was aliquoted in 12 ml glass tubes and the chloroform was evaporated under a stream of N_2 (g). To the dry lipid substrate 0.5 ml of cell free medium was added and 25 ul 1.0 M potassium phosphate, pH 7.2. The reaction mixture was sonicated in a water bath (Branson 2510) for 30 min and 30 was further incubated at 37 °C to a final incubation of 20 hours. The synthesised wax esters were extracted and quantified as described above. The results are presented in table 1 and clearly show that apart from hexacosanol all alcohols could efficiently be used as acyl group acceptors in the synthesis of wax esters. Hexacosanol is a saturated 26 carbon alcohol with a high melting point and is 35 therefore badly emulsified at assay conditions used in the present study and this is suggested to be the main reason to that only 5 % of added radiolabeled acylgroups were esterified with the hexacosanol. In contrast, approximately 40 to 50 percent of the added radiolabeled acyl group formed wax esters with the other alcohols tested (table1). The ricinoleic acid contains a hydroxyl group at position 12 in the carbon

chain, however it could not act as an acyl acceptor in the catalyses of wax esters by the ATWAX enzyme. It is therefore concluded that in the synthesis of waxesters from lecithin and ricinoleoyl alcohol as shown in table 1 the acylgroups derived from the lecithin is exclusively esterified to the hydroxyl group of position 1 and not to that of position 12 of the ricinoleoyl alcohol.

Table 1

Synthesis of wax esters from *sn1*-oleoyl-*sn2*-[^{14}C]oleoyl-phosphatidylcholine and different alcohols (acyl acceptors) in cell free supernatants.

Acyl acceptor	**[^{14}C]-acylgroups in wax esters (% of added)**
Butanol	Nd
Decanol	42,8
Hexadecanol	44,8
13c-Docosenol	53,9
Hexacosanol	5,1
Ricinoleoyl alcohol	51,1
Ricinoleoyl fatty acid	nd

EXAMPLE 6

The ability to catalyse the synthesis of wax ester with the membrane bound full length PDAT expressed in *Saccharomyces cerevisiae* was examined. Microsomes were prepared from wild type *S. cerevisiae* cells overexpressing the yeast PDAT gene *LRO1* as described in, Dahlqvist et al., 2000 (PNAS 97:6487-6492) and were assayed for wax ester synthesis. The PDAT activity was analysed with the addition of the lipid substrates dissolved in benzene to dry aliquots of lyophilised microsomes (corresponding to 12 nmol of microsomal phosphatidylcholine) (Dahlqvist et al., 2000, PNAS 97:6487-6492). As substrate we used 2.5 nmol of *sn*1-oleoyl – *sn*2 – [^{14}C]oleoyl-phosphatidylcholine (lane 1) together with either 2.5 nmol ricinoleoyl alcohol (Ric-OH, lane 2) or 13c-docosenol (22:1-OH, lane 3). The enzymatic assay and lipid analysis were performed as described in Dahlqvist et al., 2000 (PNAS 97:6487 – 6492). It is clearly shown in figure 6 that triacylglycerols are synthesised by an acyltransfer of the radiolabeled acyl group of the added phospholipid to the endogenous diacylglycerols present in the microsomal preparation (Fig 6, lane 1), as previously reported. However, adding ricinoleoyl alcohol or 13c-docosenol (Fig 6, lane 2 and 3) to the incubation formed no detectable amounts of waxesters. It is therefore concluded that the full-length

membrane associated PDAT encoded by the *LRO1* gene do not catalyse the wax ester synthesis such as presently shown above for the membrane independent acyltransferase, ATWAX. Hence it is here shown that the utilisation of different acyl donors and acceptors by an membrane independent acyltransferase is less
5 limited, as compared to acyltransferases integrated into lipidmembranes via one or several membrane-spanning regions, since the accessibility of different substrates are restricted to the vicinity of the localisation of the membrane integrated enzyme.

EXAMPLE 7

10

Genes homologous to the *Saccharomyces cerevisiae* gene *LRO1*.

The yeast PDAT (ScPDAT) amino acid sequence encoded by the *LRO1* gene was used to search the NCBI databases for homologous sequences in plants and microbes. In *Schizosaccharomyces pombe* one gene SpPDAT with strong
15 homologies to the yeast PDAT gene *LRO1* was identified. Four *Arabidopsis thaliana* genes At6704, At1254, At3027 and At4557 with clear homology to amino acid sequence encoded by the yeast *LRO1* gene were identified. Additionally two plant genes Cp6704 and Cp1254 homologies to At6704 and At1254, respectively, were identified in *Crepis palaestina*. The full-length genes of Cp6704 and Cp1254
20 were amplified from double stranded cDNA, synthesised with *C. palaestina* seed mRNA as template. The coding region of the SpPDAT, At6704, At1254, At3027, At4557, Cp6704 and Cp1254 nucleic acid sequences are shown in SEQ ID NO:16, 4, 12, 8, 10, 18 and 20. The amino acid sequences encoded by SpPDAT, At6704, At1254, At3027, At4557 Cp6704 and Cp1254 sequences, i.w., SEQ ID NO:17, 5,
25 13, 9, 19 and 21, are aligned together with the yeast *LRO1* in Figure 7 using hierarchical clustering as described in F. Corpet, 1988, Nucl. Acids Res., 16; 10881-10890. In similarity with the yeast PDAT ScPDAT, as predicted by the THMM2.0 program (A. Krogh, B. Larsson, G. von Heijne, and E. L. L. Sonnhammer, (2001) Journal of Molecular Biology, 305:567-580), all these plant genes contains a single
30 N terminal localised transmembrane spanning region as marked with gray boxes in Figure 7. The full length At6704 gene has been shown to encode an enzyme with PDAT activity (Banas et al., 2003 in Advanced Research on Plant Lipids 179 - 182). Any data on the activity associated with the gene products of At1254, At3027 or the At4557 has not yet been published.

35 Additionally, nucleotide sequences from *Aspergillus nidulans* and *Aspergillus fumigatus* (SEQ ID NO 14 and 15) were identified. Translated amino acid sequences from these nucleotide sequences shows strong homologies with the amino acid sequence of the yeast PDAT (Figure 8). In similarity with the yeast

PDAT the *Aspergillus* sequences contains a single N-terminal membrane-spanning region, within the first 100 amino acids, as predicted by the THMM2.0 program.

5

EXAMPLE 8

Expression of an active membrane independent acyltransferase from a nucleotide
10 sequence, derived from the plant gene At6704.

The yeast PDAT protein sequence was used to search the NCBI databases for homologous sequences in *Arabidopsis thaliana*. One of the identified sequences was At6704 encoding a plant PDAT (Banas et al., 2003 in Advanced Research on Plant Lipids 179 - 182). A cDNA clone, corresponding to At6704 was ordered from
15 the AIMS database. The clone was sequenced and found to contain an insertion of one base. This extra base was deleted through site directed mutagenesis. The At6704 gene encodes a plant-PDAT with a membrane-spanning region from aa 48 to aa 70 as predicted by the THMM2.0 program (A. Krogh, B. Larsson, G. von Heijne, and E. L. L. Sonnhammer, (2001) Journal of Molecular Biology, 305:567-
20 580).

A nucleotide sequence, SEQ ID NO: 6 identical in sequence to the part of the plant PDAT gene At6704 encoding the amino acid residues 74 to 671 (SEQ ID NO: 7) was amplified by thermo stable Pfu Turbo Polymerase (Stratagene Inc.) from the plasmid template pUS56 containing the full length At6704 plant gene. The forward
25 (5' end) primers used for this PCR reaction was;
5′CCATGGGAATGAATTCGCAATGCCTGCGAGCTTCCCTCAGTATGTA3′
The oligonucleotide sequence;
5'GAATTCGTTAGCGGCCGCCAGCTTCAGGTCAATACGCTCCGACCA3'
was used as the reverse (3' end) primer in the PCR amplification.
30 Th amino acid sequence (SEQ ID NO:7) encoded by the amplified nucleotide sequence (SEQ ID NO:6) lacks the amino acid residues 1 to amino acid 73 including the transmembrane region of amino acid residues 48 to 70 present in the plant PDAT as predicted by the THMM2.0 program.

The amplified PCR product above, was purified, digested by EcoRI and NotI,
35 and subcloned between the EcoRI and NotI sites of the Pichia expression vector PpicZαA in frame with the N-terminal sequence encoding the αfactor signalpeptide present in the expression vector and a C-terminal c-myc epitope followed by a polyhistidine tag. The resultant Pichia expression vectors are named pHisATWAX-P6. The vector was linearized using unique SacI restriction site for transformation

into *Pichia pastoris* host strain KM71H. Transformants were cultivated for the expression of the transformed gene as described in EXAMPLE 2.To determine the enzyme activity in the cell free culture medium the cell free supernatant was assayed for enzyme activity as follows. The cell free supernatant of the induced
5 cultures of KM71H transformed with pHisATWAX as described in EXAMPLE 2, from 116 hours of growth of *Pichia pastoris* KM71H transformed with the pHisATWAX–P6 secreting, the polypeptide HisATWAX-P6 (Fig 9, lane 1 and 2), the congenic wt strain (Fig 9, lane 3 and 4) and KM71H expressing the polypeptide ATWAX were assayed for acyl transferase activity.

10 .Lipid substrate, *sn1*-palmitoyl *sn2*-[^{14}C]linoleoyl- phosphatidylethanolamine (5 nmol; 5000 dpm/ nmol) and dioleoylglycerol (2.5 nmol) dissolved in chloroform was aliquoted in 1.5 ml tubes and the chloroform was evaporated under a stream of N_2 (g). After addition of 20 ul 0.25 M potassium phosphate, pH 7.2, the mixture was violently agitated and 80 ul of cell free supernatant was added and incubated at
15 30°C for 90 min. Lipids were extracted from the reaction mixture into chloroform (Bligh, E.G. and Dyer, W.J (1959) Can. J. Biochem. Physiol. 37, 911-917) and separated by TLC on silica gel 60 plates (200 x 200 mm) in chloroform / methanol / acetic acid / water (85:15:10:3.5) migrating 90 mm using an automatic developing chamber (Camag). The plate was dried and redeveloped in hexane / diethyl ether /
20 acetic acid (80:20:1) with a solvent migration of 180 mm. The radioactive lipids were visualized and quantified on the plates by electronic autoradiography (Instant Imager; Packard). As a control, the enzyme activity in the cell free supernatant of the wild type host strain culture was analysed. As shown in Figure 9, radiolabeled triacylglycerol (TAG) is formed, from the added lipid substrate, *sn1*-palmitoyl *sn2*-
25 [^{14}C]linoleoyl- phosphatidylethanolamine and dioleoylglycerol, in cell free extract of Pichia strain transformed with pHisATWAX-P6. This demonstrates that the truncated membrane independent form of the plant PDAT, referred to as a membrane independent acyltransferase that we have named HisATWAX-P6, is able of catalysing the formation of TAG by an acyltransfer from
30 phosphatidylethanolamine to diacylglycerol (DAG).

CLAIMS

1. A nucleotide sequence, derived from a nucleotide sequence encoding an acyltransferase polypeptide comprising at least one membrane-spanning region, encoding an improved active membrane independent acyltransferase polypeptide in which at least one amino acid residue of the membrane-spanning region has been deleted and/or substituted as compared to the original acyltransferase polypeptide, wherein the encoded active membrane independent acyltransferase polypeptide can produce fatty acid esters and/or fatty acid thioesters such as triacylglycerols, diacylglycerols, monoacylglycerols, phospholipids, glycolipids, lysolipids, waxesters, acylated carbohydrates and acylated amino acids.
2. The nucleotide sequence according to claim 1, wherein the nucleotide sequence is derived from a nucleotide sequence encoding a lipid dependent acyltransferase polypeptide.
3. The nucleotide sequence according to any of preceding claims, wherein the nucleotide sequence is derived from a nucleotide sequence encoding a lipid dependent acyltransferase polypeptide catalysing an acyl transfer reaction in which acylphospholipids acts as acyl donors.
4. The nucleotide sequence according to any of preceding claims, wherein the nucleotide sequence is derived from a nucleotide sequence encoding a phospholipid: diacylglycerol acyltransferase.
5. The nucleotide sequence according to any of the preceding claims, wherein the nucleotide sequence is derived from a nucleotide sequence shown in SEQ ID NO:1, 4, 8, 10, 12, 14, 15, 16, 18 or 20.
6. The nucleotide sequence according to claim 5, wherein from 1 to 291 nucleotide sequence residue(s) of the 5'-end has/have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:1.
7. The nucleotide sequence according to claim 6, wherein at least the nucleotide sequence residues 238 to 288 have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:1.
8. The nucleotide sequence according to claim 5, wherein from 1 to 219 nucleotide sequence residue(s) of the 5'-end has/have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO: 4, 1-87 nucleotide sequence residue(s) of SEQ ID NO:8 and SEQ ID NO:10 and 1-190 nucleotide sequence residue(s) of SEQ ID NO:12.
9. The nucleotide sequence according to claim 8, wherein at least the nucleotide sequence residues 142 to 210 have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:4, 19-87 nucleotide sequence

residues of SEQ ID NO 8 and SEQ ID NO:10 and 130-190 nucleotide sequence residues of SEQ ID NO:12.

10. The nucleotide sequence according to claim 5, wherein from 1 to 228 nucleotide sequence residue(s) of the 5'-end has/have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO: 16, 1-219 nucleotide sequence residue(s) of SEQ ID NO:18 and 1-261 nucleotide sequence residue(s) of SEQ ID NO:20.
11. The nucleotide sequence according to claim 10, wherein at least the nucleotide sequence residues 169 to 228 have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:16, 151-219 nucleotide sequence residue(s) of SEQ ID NO:18 and 193-261nucleotide sequence residue(s) of SEQ ID NO:20.
12. The nucleotide sequences according to any of preceding claims, wherein the nucleotide sequences hybridises under stringent conditions to a nucleotide sequence shown in SEQ ID NO: 1, 2, 4, 6, 8, 10, 12, 14, 15, 16, 18 or 20.
13. The nucleotide sequences according to any of preceding claims, wherein the nucleotide sequence of said nucleotide sequence differs from the coding sequence of the nucleotide sequences shown in SEQ ID NO: 1, 2, 4, 6, 8, 10, 12, 14, 15, 16, 18 or 20 due to the degeneracy of the genetic code.
14. A nucleotide sequence molecule comprising;
    a) at least one promoter region which functions in a host, the promoter region is operably linked to
    b) at least one nucleotide sequence according to any of claims 1-13, which is operably linked to
    c) at least one non-translated region which functions in a host.
15. The nucleotide sequence molecule according to claim 14, wherein at least a signal peptide is operably linked between a) and b).
16. A vector harbouring a nucleotide sequence molecule according to any of claims 14-15.
17. The vector according to claim 16, wherein the vector is an expression vector.
18. The expression vector according to claim 17, wherein the expression vector is selected from the group consisting of 2μ plasmid and derivatives thereof, POT1 pJSO37 and pPICZ A, B or C.
19. A host cell comprising a nucleotide sequence molecule or a vector according to any of claims 14-18.
20. The host cell according to claim 19, wherein the host cell is a host cell capable of producing a glycosylated polypeptide.
21. The host cell according to any of claims 15-16, wherein the host cell is selected from the group consisting of fungi, yeast, insects and mammalian cells.

22. The host cell according to claim 21, wherein the host cell is selected from the group consisting of *Aspergillus, Fusarium, Trichoderma, Saccharomyces, Schizosaccharomyces, Klyveromyces, Pichia, Hansenula, Mucor* or *Yarrowia*.
23. A method for the production of an improved active membrane independent
5 acyltransferase polypeptide comprising the steps of
   i) providing a host cell according to claim 19-22 and a growth medium preparing a host cell culture;
   ii) culturing the host cell culture and
   iii) harvesting the host cell culture and recovering the improved active
10 membrane independent acyltransferase polypeptide.
24. The method according to claim 23, wherein the method is used in a fermenter.
25. The method according to claims 23-24, wherein the polypeptide is recovered either from the culture medium, the cells or after separating the cells from the culture medium.
15 26. A polypeptide obtained by the method according to any of claims 23-25, wherein the polypeptide at least is an improved active membrane independent acyltransferase polypeptide.
27. The polypeptide according to claim 26, wherein the polypeptide is an acyltransferase active at a pH range of from about 4 to about 10 and stable at a
20 temperature below 60 °C.
28. The polypeptide according to claim 27, wherein the polypeptide is an acyltransferase active at a pH of 7.2 at a temperature of about 30 °C.
29. The polypeptide according to any of claims 26-28, wherein the polypeptide at least show 75 %, 80%, 85%, 90% or 95% homology to the amino acid sequence
25 shown in SEQ ID NO:3, 7, 9, 11, 13, 17, 19, 21or a homologue thereof.
30. The polypeptide according to any of claims 26-29, wherein the polypeptide is immobilised to a carrier.
31. The polypeptide according to any of claims 26-30, wherein the polypeptide is lyophilised and/or freeze-dried.
30 32. An oligonucleotide specifically hybridising under stringent conditions to a nucleotide sequence of any one of claims 1 to 13.
33. A kit comprising the polypeptide according to any of claims 26-31 and a stabiliser.
34. The kit according to claim 33, wherein the polypeptide is in a lyophilised form
35 or freeze-dried.
35. Use of a nucleotide sequence according to any of claims 1-13 and/or a nucleotide sequence molecule according to any of claims 14-15 and/or a vector according to any of claims 16-18 and/or a host cell according to any of claims 19-22 and/or the method according to any of claims 23-25 and/or the

polypeptide according to any of claims 26-31 and/or the oligonucleotide according to claim 32 and/or the kit according to any of claims 33-34.

5

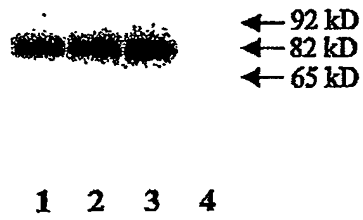


Figure 1/9

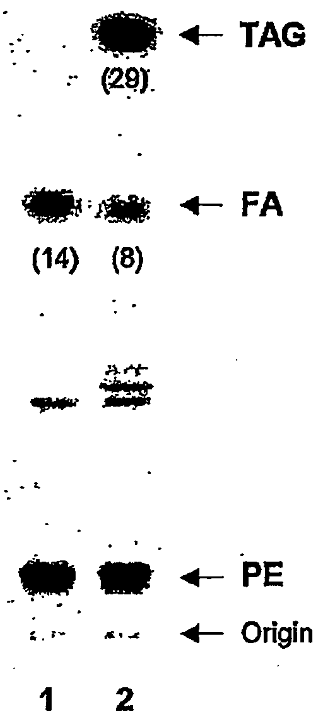


Figure 2/9

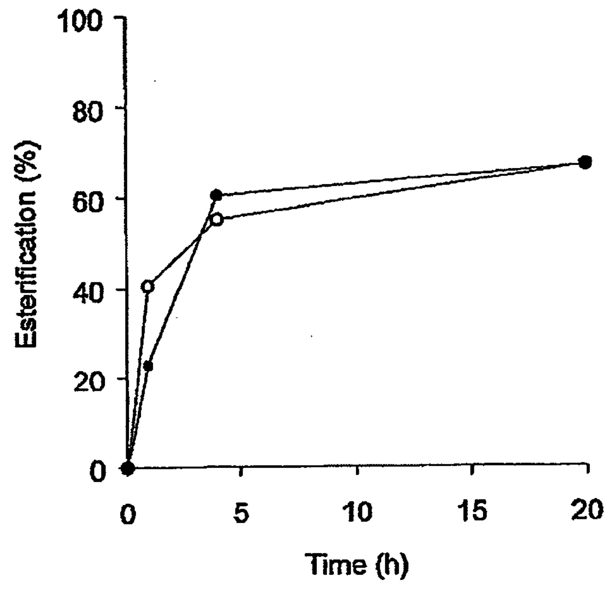
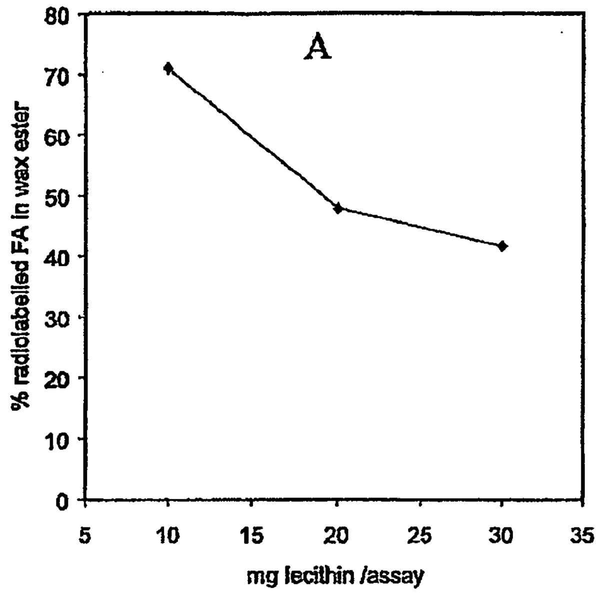


Figure 3



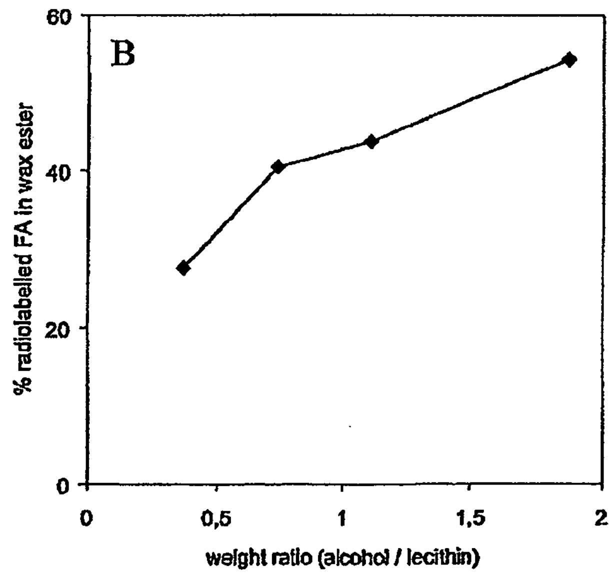


Figure 4

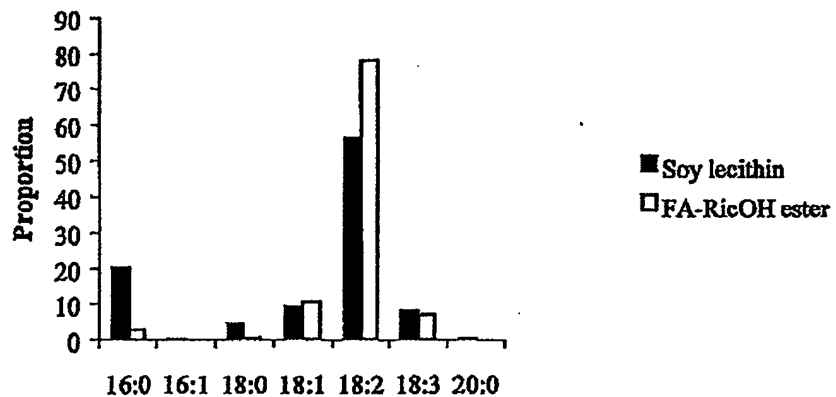


Figure 5/9

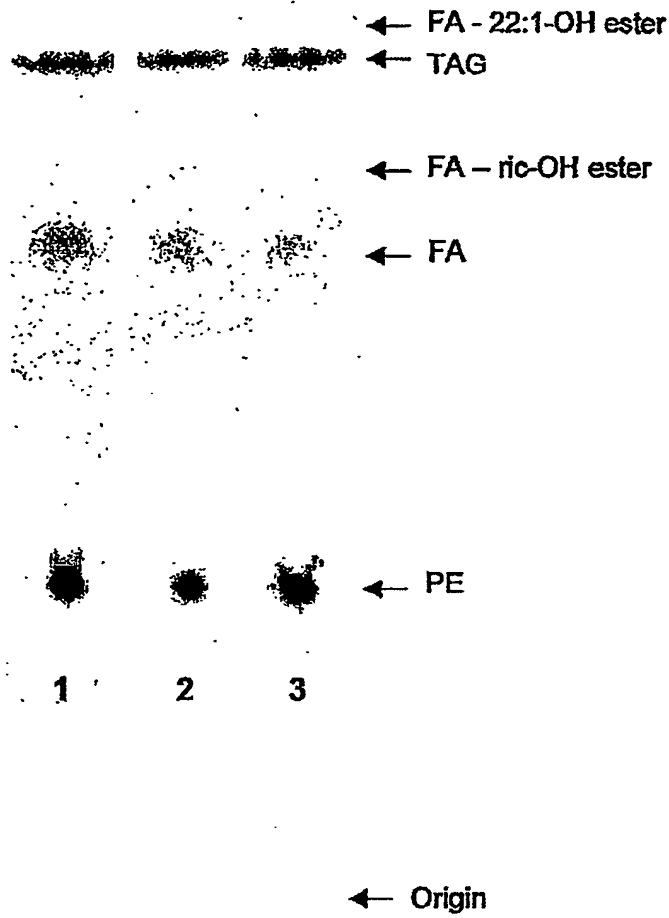


Figure 6/9

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           1                                                                                                                         130
ScPDAT     MGTLFRRNVQ NQKSDSDENN KGGSVENKRE SRNHIHHQQG LGHKRRRGIS GSAKRNERGK DFDRKRDGNG RKRWRDSRRL IFILGAFLGV LLPFSFGAYH VHNS-DSDLF DNFVNFDSLK VYLDDWKDVL
SpPDAT                    MASSK KSKTHKKKKE VKSPIDLPNS KKPTRALSEQ PSASETQSVS NKSRKS---- ----KFGKRL NFILGAILGI CGAFFFAVGD DNAVFDPATL DKFGNMLGSS DLFDDIKGYL
At6704                                                        MP LIHRKK-PTE KPSTPP---- ------SEEV VHDEDSQKKP HESSKSHHKK SNGGGKWSCI DSCCWFIGCV CVTWWFLLFL
Cp6704                                                        NA LTRRRKQPED ETQQEP---- ------DPKP DQEDDKEEKA LKRSGKKDKS KN----YSCL DNCCWFVGCV CTAWWLLLFL
At1254                                                              MS PLLRFR---- ------KLSS FSEDTINPKP KQSATVEKPK RRRSGRCSCV DSCCWLIGYL CTAWWLLLFL
Cp1254                                    MAA ILRFRKLCFV EPAHKNYGSP ETQKDE---- ------KQSL DRKQEKSQAI KEQDIDKNKK KKQVRENRCV DSCCWFIGCL CFTWWLLLFV
At3027
At4557                                                                                                                                      MKK
Consensus  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... d......... .........l

           131                                                                                                                       260
ScPDAT     PQGISSFIDD IQAGNYSTSS LDDLSENFAV GKQLLRDYNI EAKHPVVMVP GVISTGIESW GVIGDDECDS SAHFRKRLWG SFYMLRTMVM DKVCWLKHVM LDPETGLDPP NFTLRAAQGF ESTDYFIAGY
SpPDAT     SYNV--FKDA PFTTDKPSQS PS---GNEVQV GLDMYNE-GY RSDHPVIMVP GVISSGLESW SF---NNC-S IPYFRKRLWG SWSMLKAMFL DKQCWLEHLM LDKKTGLDPK GIKLRAAQGF EAADFFITGY
At6704     YNANFASFPQ YVTERITGPL PD------PP GVKLKKE-GL KAKHPVVFIP GIVTGGLELW EG---KQC-A DGLFRKRLWG G--TFGEVYK RPLCWVEHMS LDNETGLDPA GIRVRAVSGL VAADYFAPGY
Cp6704     YNANEASFPQ FVTERITGPL PD------PP GVKCLKE-GL KVKHPVVCVP GIVTGGLELW EG---HQC-N DGLFRKRLWG G--TFGEVYK RPSCWVQHMS LDNKTGMDPP GIRVRPVSGL VAADYFAPGY
At1254     YHSVPVP-AN LQA------- PE------SP GTRLSRD-GV KAFHPVILVP GIVTGGLELW EG---RPC-A EGLFRKRLWG A--SFSEILR RPLCWLEHLS LDSETGLDPS GIRVRAVPGL VAADYFAPCY
Cp1254     YHCLPAHLAG FKG------- PE------PP GVRLKNE-GL TPLHPVVLVP GIVTGGLELW EG---QPC-S EGLFRKRLWG G--SFTEILE RPLCWLEHLS LDNETGLDPP GIRVRPVPGL VAADYFAPGY
At3027       MGANSKS VYASETVIAV ESLIGGGRIA VEDETEFHGD YSKLSGIIIP GFASTQLRAW SI---LDCPY TPLDFNPLDL VWLDTTKLLS AVNCWFKCMV LDPYNQTDHP ECKSRPDSGL SAITELDPGY
At4557     ISSHYSVVIA ILVVVIMTSH CQAVGGNVYP LILVPGNGGN QLEVRLDREY KPSSVWCSSW LYPIHKKSGG WFRLWFDAAV LLSPFTRCFS DRMMLYYDPD LDDYQNAPGV QTRVPHFGST KSLLYLDPRL
onsensus   .......... .......... .........p g.......g. ...hpv..vp g..s.gle.. .......c.. ..lfrkrlwg ....f..... ...cw..h.. l...tgldp. ..rvr...gl .a.dyf.pgy

           261                                                                                                                       390
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Consensus  .......w. v...nl..ig y...m..a. ydw.l....s ....evrdq. l.. ..i. ....nggkkv v...hsmg.. .flh.lkwve a.....g.gg .p.w..kh.k a..n.g.p.l g.pkav..ll

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Consensus  gge.kd.... ..la...l.. .....q.l.. ..km..t... ..s.lpkg.. .......... e......... ......n... .......... ........y gr..sf.... ..d....... ..........
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Figure 7A/9

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onsensus  .......... .......... ....i..... .......... ......a.k. .......... e.g....l.. .......... w.np.e..lp .ap.m.iy.. .gv..pter. y.y....... ..........

          651                                                                                                                   778
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Figure 7B/9

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AnPDAT
AtPDAT
Consensus

141 ... 260

ScPDAT
AnPDAT
AtPDAT
Consensus

261 ... 390

Figure 8/9

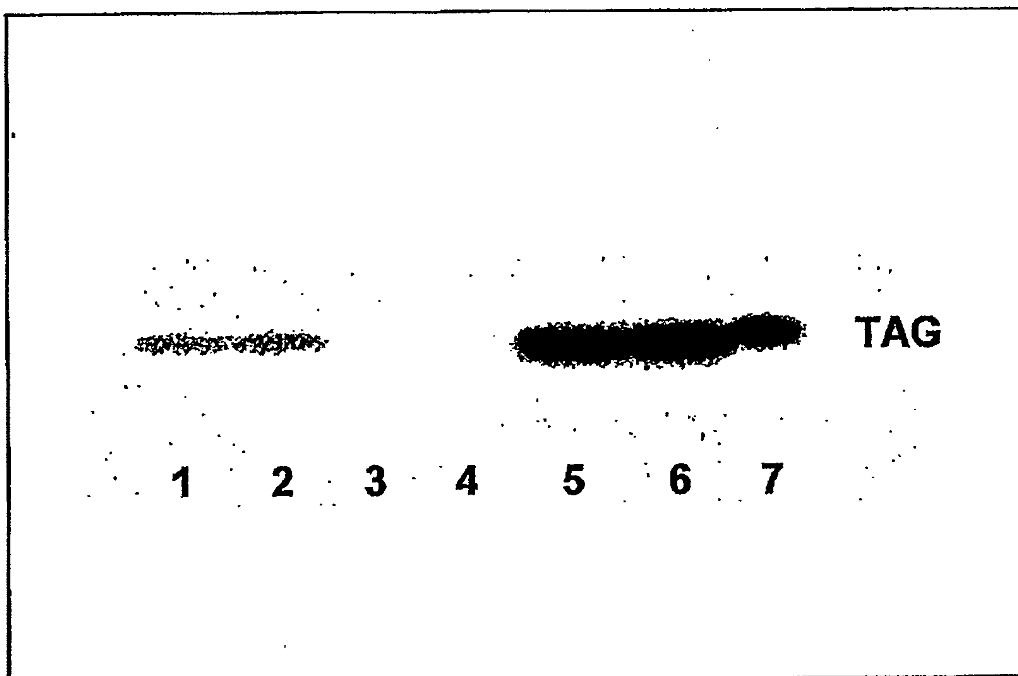

Figure 9/9

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Val Leu Ile Ala Asn Leu Ala His Ile Gly Tyr Glu Glu Lys Asn Met
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Tyr Met Ala Ala Tyr Asp Trp Arg Leu Ser Phe Gln Asn Thr Glu Val
210 215 220

Arg Asp Gln Thr Leu Ser Arg Met Lys Ser Asn Ile Glu Leu Met Val
225 230 235 240

Ser Thr Asn Gly Gly Lys Lys Ala Val Ile Val Pro His Ser Met Gly
245 250 255

Val Leu Tyr Phe Leu His Phe Met Lys Trp Val Glu Ala Pro Ala Pro
260 265 270

Leu Gly Gly Gly Gly Gly Pro Asp Trp Cys Ala Lys Tyr Ile Lys Ala
275 280 285

Val Met Asn Ile Gly Gly Pro Phe Leu Gly Val Pro Lys Ala Val Ala
290 295 300

Gly Leu Phe Ser Ala Glu Ala Lys Asp Val Ala Val Ala Arg Ala Ile
305 310 315 320

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Ala Pro Gly Phe Leu Asp Thr Asp Ile Phe Arg Leu Gln Thr Leu Gln
                325                 330                 335

His Val Met Arg Met Thr Arg Thr Trp Asp Ser Thr Met Ser Met Leu
            340                 345                 350

Pro Lys Gly Gly Asp Thr Ile Trp Gly Gly Leu Asp Trp Ser Pro Glu
        355                 360                 365

Lys Gly His Thr Cys Cys Gly Lys Lys Gln Lys Asn Asn Glu Thr Cys
    370                 375                 380

Gly Glu Ala Gly Glu Asn Gly Val Ser Lys Lys Ser Pro Val Asn Tyr
385                 390                 395                 400

Gly Arg Met Ile Ser Phe Gly Lys Glu Val Ala Glu Ala Ala Pro Ser
                405                 410                 415

Glu Ile Asn Asn Ile Asp Phe Arg Gly Ala Val Lys Gly Gln Ser Ile
            420                 425                 430

Pro Asn His Thr Cys Arg Asp Val Trp Thr Glu Tyr His Asp Met Gly
        435                 440                 445

Ile Ala Gly Ile Lys Ala Ile Ala Glu Tyr Lys Val Tyr Thr Ala Gly
    450                 455                 460

Glu Ala Ile Asp Leu Leu His Tyr Val Ala Pro Lys Met Met Ala Arg
465                 470                 475                 480

Gly Ala Ala His Phe Ser Tyr Gly Ile Ala Asp Asp Leu Asp Asp Thr
                485                 490                 495

Lys Tyr Gln Asp Pro Lys Tyr Trp Ser Asn Pro Leu Glu Thr Lys Leu
            500                 505                 510

Pro Asn Ala Pro Glu Met Glu Ile Tyr Ser Leu Tyr Gly Val Gly Ile
        515                 520                 525

Pro Thr Glu Arg Ala Tyr Val Tyr Lys Leu Asn Gln Ser Pro Asp Ser
    530                 535                 540

Cys Ile Pro Phe Gln Ile Phe Thr Ser Ala His Glu Glu Asp Glu Asp
545                 550                 555                 560
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Ser Cys Leu Lys Ala Gly Val Tyr Asn Val Asp Gly Asp Glu Thr Val
565 570 575

Pro Val Leu Ser Ala Gly Tyr Met Cys Ala Lys Ala Trp Arg Gly Lys
580 585 590

Thr Arg Phe Asn Pro Ser Gly Ile Lys Thr Tyr Ile Arg Glu Tyr Asn
595 600 605

His Ser Pro Pro Ala Asn Leu Leu Glu Gly Arg Gly Thr Gln Ser Gly
610 615 620

Ala His Val Asp Ile Met Gly Asn Phe Ala Leu Ile Glu Asp Ile Met
625 630 635 640

Arg Val Ala Ala Gly Gly Asn Gly Ser Asp Ile Gly His Asp Gln Val
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His Ser Gly Ile Phe Glu Trp Ser Glu Arg Ile Asp Leu Lys Leu
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<212> DNA
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ttcattcctg ggattgtcac cggtgggctc gagctttggg aaggcaaaca atgcgctgat 180

ggtttattta gaaaacgttt gtggggtgga acttttggtg aagtctacaa aaggcctcta 240

tgttgggtgg aacacatgtc acttgacaat gaaactgggt tggatccagc tggtattaga 300

gttcgagctg tatcaggact cgtggctgct gactactttg ctcctggcta ctttgtctgg 360

gcagtgctga ttgctaacct tgcacatatt ggatatgaag agaaaaatat gtacatggct 420

gcatatgact ggcggctttc gtttcagaac acagaggtac gtgatcagac tcttagccgt 480

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aaggatgttg cagttgccag agcgattgcc ccaggattct tagacaccga tatatttaga 780

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cttcagacct tgcagcatgt aatgagaatg acacgcacat gggactcaac aatgtctatg    840
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<213>  Arabidopsis thaliana

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Thr Gly Pro Leu Pro Asp Pro Pro Gly Val Lys Leu Lys Lys Glu Gly
            20                  25                  30

Leu Lys Ala Lys His Pro Val Val Phe Ile Pro Gly Ile Val Thr Gly
        35                  40                  45

Gly Leu Glu Leu Trp Glu Gly Lys Gln Cys Ala Asp Gly Leu Phe Arg
    50                  55                  60

Lys Arg Leu Trp Gly Gly Thr Phe Gly Glu Val Tyr Lys Arg Pro Leu
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65 70 75 80

Cys Trp Val Glu His Met Ser Leu Asp Asn Glu Thr Gly Leu Asp Pro
85 90 95

Ala Gly Ile Arg Val Arg Ala Val Ser Gly Leu Val Ala Ala Asp Tyr
100 105 110

Phe Ala Pro Gly Tyr Phe Val Trp Ala Val Leu Ile Ala Asn Leu Ala
115 120 125

His Ile Gly Tyr Glu Glu Lys Asn Met Tyr Met Ala Ala Tyr Asp Trp
130 135 140

Arg Leu Ser Phe Gln Asn Thr Glu Val Arg Asp Gln Thr Leu Ser Arg
145 150 155 160

Met Lys Ser Asn Ile Glu Leu Met Val Ser Thr Asn Gly Gly Lys Lys
165 170 175

Ala Val Ile Val Pro His Ser Met Gly Val Leu Tyr Phe Leu His Phe
180 185 190

Met Lys Trp Val Glu Ala Pro Ala Pro Leu Gly Gly Gly Gly Gly Pro
195 200 205

Asp Trp Cys Ala Lys Tyr Ile Lys Ala Val Met Asn Ile Gly Gly Pro
210 215 220

Phe Leu Gly Val Pro Lys Ala Val Ala Gly Leu Phe Ser Ala Glu Ala
225 230 235 240

Lys Asp Val Ala Val Ala Arg Ala Ile Ala Pro Gly Phe Leu Asp Thr
245 250 255

Asp Ile Phe Arg Leu Gln Thr Leu Gln His Val Met Arg Met Thr Arg
260 265 270

Thr Trp Asp Ser Thr Met Ser Met Leu Pro Lys Gly Gly Asp Thr Ile
275 280 285

Trp Gly Gly Leu Asp Trp Ser Pro Glu Lys Gly His Thr Cys Cys Gly
290 295 300

Lys Lys Gln Lys Asn Asn Glu Thr Cys Gly Glu Ala Gly Glu Asn Gly
305 310 315 320

```
Val Ser Lys Lys Ser Pro Val Asn Tyr Gly Arg Met Ile Ser Phe Gly
                325                 330                 335

Lys Glu Val Ala Glu Ala Ala Pro Ser Glu Ile Asn Asn Ile Asp Phe
            340                 345                 350

Arg Gly Ala Val Lys Gly Gln Ser Ile Pro Asn His Thr Cys Arg Asp
        355                 360                 365

Val Trp Thr Glu Tyr His Asp Met Gly Ile Ala Gly Ile Lys Ala Ile
    370                 375                 380

Ala Glu Tyr Lys Val Tyr Thr Ala Gly Glu Ala Ile Asp Leu Leu His
385                 390                 395                 400

Tyr Val Ala Pro Lys Met Met Ala Arg Gly Ala Ala His Phe Ser Tyr
                405                 410                 415

Gly Ile Ala Asp Asp Leu Asp Asp Thr Lys Tyr Gln Asp Pro Lys Tyr
            420                 425                 430

Trp Ser Asn Pro Leu Glu Thr Lys Leu Pro Asn Ala Pro Glu Met Glu
        435                 440                 445

Ile Tyr Ser Leu Tyr Gly Val Gly Ile Pro Thr Glu Arg Ala Tyr Val
    450                 455                 460

Tyr Lys Leu Asn Gln Ser Pro Asp Ser Cys Ile Pro Phe Gln Ile Phe
465                 470                 475                 480

Thr Ser Ala His Glu Glu Asp Glu Asp Ser Cys Leu Lys Ala Gly Val
                485                 490                 495

Tyr Asn Val Asp Gly Asp Glu Thr Val Pro Val Leu Ser Ala Gly Tyr
            500                 505                 510

Met Cys Ala Lys Ala Trp Arg Gly Lys Thr Arg Phe Asn Pro Ser Gly
        515                 520                 525

Ile Lys Thr Tyr Ile Arg Glu Tyr Asn His Ser Pro Pro Ala Asn Leu
    530                 535                 540

Leu Glu Gly Arg Gly Thr Gln Ser Gly Ala His Val Asp Ile Met Gly
545                 550                 555                 560
```

```
Asn Phe Ala Leu Ile Glu Asp Ile Met Arg Val Ala Ala Gly Gly Asn
                565                 570                 575

Gly Ser Asp Ile Gly His Asp Gln Val His Ser Gly Ile Phe Glu Trp
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Ser Glu Arg Ile Asp Leu Lys Leu
        595                 600


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<211>  1902
<212>  DNA
<213>  Arabidopsis thaliana

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ctatcgggta taatcattcc gggatttgcg tcgacgcagc tacgagcgtg gtcgatcctt     180

gactgtccat acactccgtt ggacttcaat ccgctcgacc tcgtatggct agacaccact     240

aagcttcttt ctgctgtcaa ctgctggttt aagtgtatgg tgctagatcc ttataatcaa     300

acagaccatc ccgagtgtaa gtcacggcct gacagtggtc tttcagccat cacagaattg     360

gatccaggtt acataacagg tcctctttct actgtctgga aagagtggct taagtggtgt     420

gttgagtttg gtgtagaagc aaatgcaatt gtcgctgttc catacgattg gagattgtca     480

ccaaccaaat tggaagagcg tgacctttac tttcacaagc tcaagttgac ctttgaaact     540

gctttaaaac tccgtggcgg cccttctata gtatttgccc attcaatggg taataatgtc     600

ttcagatact ttctggaatg gctgaggcta gaaattgcac caaaacatta tttgaagtgg     660

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cggttgttgt ccaattcttt tgcgtcgtca ttgtggctta tgccattttc aaagaattgc     840

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attaacattg aaattccttc cactagcgtt acagaaacag ctctagtcaa catgaccagc    1020

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cttttcaaag caatagaaga ctatgaccca gatagcaaga ggatgttaca ccagttaaag    1140

aagttgtatc atgatgaccc tgtttttaat cctctgactc cttgggagag accacctata    1200

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ccaagtggca aaccttatcc tgataattgg atcatcacgg atatcattta tgaaactgaa   1320
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gatgagacgg taccctatca ttcactctct tggtgcaaga attggctcgg acctaaagtt   1440
aacataacaa tggctcccca gccagaacac gatggaagcg acgtacatgt ggaactaaat   1500
gttgatcatg agcatgggtc agacatcata gctaacatga caaaagcacc aagggttaag   1560
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cttgataaaa caaatcacag gaacatagta agatctccag ttctgatgag ggagttatgg   1680
cttcagatgt ggcatgacat tcagcctggt gcaaagtcca aatttgtcac caaagccaag   1740
cgcgggccac ttagagatgc ggattgctac tgggattacg ggaaagcctg ttgtgcttgg   1800
caagaatact gtgaatacag atacagtttt ggggatgttc acttaggaca aagttgtaga   1860
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<210>  9
<211>  633
<212>  PRT
<213>  Arabidopsis thaliana

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Val Phe Phe Leu Ile Cys Gly Gly Arg Thr Ala Val Glu Asp Glu Thr
            20                  25                  30

Glu Phe His Gly Asp Tyr Ser Lys Leu Ser Gly Ile Ile Ile Pro Gly
        35                  40                  45

Phe Ala Ser Thr Gln Leu Arg Ala Trp Ser Ile Leu Asp Cys Pro Tyr
    50                  55                  60

Thr Pro Leu Asp Phe Asn Pro Leu Asp Leu Val Trp Leu Asp Thr Thr
65                  70                  75                  80

Lys Leu Leu Ser Ala Val Asn Cys Trp Phe Lys Cys Met Val Leu Asp
                85                  90                  95

Pro Tyr Asn Gln Thr Asp His Pro Glu Cys Lys Ser Arg Pro Asp Ser
            100                 105                 110

Gly Leu Ser Ala Ile Thr Glu Leu Asp Pro Gly Tyr Ile Thr Gly Pro
        115                 120                 125
```

```
Leu Ser Thr Val Trp Lys Glu Trp Leu Lys Trp Cys Val Glu Phe Gly
    130                 135                 140

Val Glu Ala Asn Ala Ile Val Ala Val Pro Tyr Asp Trp Arg Leu Ser
145                 150                 155                 160

Pro Thr Lys Leu Glu Glu Arg Asp Leu Tyr Phe His Lys Leu Lys Leu
                165                 170                 175

Thr Phe Glu Thr Ala Leu Lys Leu Arg Gly Gly Pro Ser Ile Val Phe
            180                 185                 190

Ala His Ser Met Gly Asn Asn Val Phe Arg Tyr Phe Leu Glu Trp Leu
        195                 200                 205

Arg Leu Glu Ile Ala Pro Lys His Tyr Leu Lys Trp Leu Asp Gln His
    210                 215                 220

Ile His Ala Tyr Phe Ala Val Gly Ala Pro Leu Leu Gly Ser Val Glu
225                 230                 235                 240

Ala Ile Lys Ser Thr Leu Ser Gly Val Thr Phe Gly Leu Pro Val Ser
                245                 250                 255

Glu Gly Thr Ala Arg Leu Leu Ser Asn Ser Phe Ala Ser Ser Leu Trp
            260                 265                 270

Leu Met Pro Phe Ser Lys Asn Cys Lys Gly Asp Asn Thr Phe Trp Thr
        275                 280                 285

His Phe Ser Gly Gly Ala Ala Lys Lys Asp Lys Arg Val Tyr His Cys
    290                 295                 300

Asp Glu Glu Glu Tyr Gln Ser Lys Tyr Ser Gly Trp Pro Thr Asn Ile
305                 310                 315                 320

Ile Asn Ile Glu Ile Pro Ser Thr Ser Val Thr Glu Thr Ala Leu Val
                325                 330                 335

Asn Met Thr Ser Met Glu Cys Gly Leu Pro Thr Leu Leu Ser Phe Thr
            340                 345                 350

Ala Arg Glu Leu Ala Asp Gly Thr Leu Phe Lys Ala Ile Glu Asp Tyr
        355                 360                 365
```

```
Asp Pro Asp Ser Lys Arg Met Leu His Gln Leu Lys Lys Leu Tyr His
    370                 375                 380

Asp Asp Pro Val Phe Asn Pro Leu Thr Pro Trp Glu Arg Pro Pro Ile
385                 390                 395                 400

Lys Asn Val Phe Cys Ile Tyr Gly Ala His Leu Lys Thr Glu Val Gly
                405                 410                 415

Tyr Tyr Phe Ala Pro Ser Gly Lys Pro Tyr Pro Asp Asn Trp Ile Ile
            420                 425                 430

Thr Asp Ile Ile Tyr Glu Thr Glu Gly Ser Leu Val Ser Arg Ser Gly
        435                 440                 445

Thr Val Val Asp Gly Asn Ala Gly Pro Ile Thr Gly Asp Glu Thr Val
    450                 455                 460

Pro Tyr His Ser Leu Ser Trp Cys Lys Asn Trp Leu Gly Pro Lys Val
465                 470                 475                 480

Asn Ile Thr Met Ala Pro Gln Pro Glu His Asp Gly Ser Asp Val His
                485                 490                 495

Val Glu Leu Asn Val Asp His Glu His Gly Ser Asp Ile Ile Ala Asn
            500                 505                 510

Met Thr Lys Ala Pro Arg Val Lys Tyr Ile Thr Phe Tyr Glu Asp Ser
        515                 520                 525

Glu Ser Ile Pro Gly Lys Arg Thr Ala Val Trp Glu Leu Asp Lys Thr
    530                 535                 540

Asn His Arg Asn Ile Val Arg Ser Pro Val Leu Met Arg Glu Leu Trp
545                 550                 555                 560

Leu Gln Met Trp His Asp Ile Gln Pro Gly Ala Lys Ser Lys Phe Val
                565                 570                 575

Thr Lys Ala Lys Arg Gly Pro Leu Arg Asp Ala Asp Cys Tyr Trp Asp
            580                 585                 590

Tyr Gly Lys Ala Cys Cys Ala Trp Gln Glu Tyr Cys Glu Tyr Arg Tyr
        595                 600                 605

Ser Phe Gly Asp Val His Leu Gly Gln Ser Cys Arg Leu Arg Asn Thr
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Ser Ala Asn Met Leu Leu Gln Tyr Ile
625 630

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gaccctgatt tggatgatta ccaaaatgct cctggtgtcc aaacccgggt tcctcatttc 360

ggttcgacca aatcacttct atacctcgac cctcgtctcc gagatgccac atcttacatg 420

gaacatttgg cgaaagctct agagaaaaaa tgcgggtatg ttaacgacca aaccatccta 480

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agcctagcag ctttgaaagt cgatggcttg aacaccgtag agattgatgg agtttcgcat 1200

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<211> 432
<212> PRT

<213> Arabidopsis thaliana

<400> 11

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Val Val Thr Met Thr Ser Met Cys Gln Ala Val Gly Ser Asn Val Tyr
            20                  25                  30

Pro Leu Ile Leu Val Pro Gly Asn Gly Gly Asn Gln Leu Glu Val Arg
        35                  40                  45

Leu Asp Arg Glu Tyr Lys Pro Ser Ser Val Trp Cys Ser Ser Trp Leu
    50                  55                  60

Tyr Pro Ile His Lys Lys Ser Gly Gly Trp Phe Arg Leu Trp Phe Asp
65                  70                  75                  80

Ala Ala Val Leu Leu Ser Pro Phe Thr Arg Cys Phe Ser Asp Arg Met
                85                  90                  95

Met Leu Tyr Tyr Asp Pro Asp Leu Asp Asp Tyr Gln Asn Ala Pro Gly
            100                 105                 110

Val Gln Thr Arg Val Pro His Phe Gly Ser Thr Lys Ser Leu Leu Tyr
        115                 120                 125

Leu Asp Pro Arg Leu Arg Asp Ala Thr Ser Tyr Met Glu His Leu Val
    130                 135                 140

Lys Ala Leu Glu Lys Lys Cys Gly Tyr Val Asn Asp Gln Thr Ile Leu
145                 150                 155                 160

Gly Ala Pro Tyr Asp Phe Arg Tyr Gly Leu Ala Ala Ser Gly His Pro
                165                 170                 175

Ser Arg Val Ala Ser Gln Phe Leu Gln Asp Leu Lys Gln Leu Val Glu
            180                 185                 190

Lys Thr Ser Ser Glu Asn Glu Gly Lys Pro Val Ile Leu Leu Ser His
        195                 200                 205

Ser Leu Gly Gly Leu Phe Val Leu His Phe Leu Asn Arg Thr Thr Pro
    210                 215                 220

Ser Trp Arg Arg Lys Tyr Ile Lys His Phe Val Ala Leu Ala Ala Pro
```

```
225                 230                 235                 240

Trp Gly Gly Thr Ile Ser Gln Met Lys Thr Phe Ala Ser Gly Asn Thr
                245                 250                 255

Leu Gly Val Pro Leu Val Asn Pro Leu Leu Val Arg Arg His Gln Arg
            260                 265                 270

Thr Ser Glu Ser Asn Gln Trp Leu Leu Pro Ser Thr Lys Val Phe His
        275                 280                 285

Asp Arg Thr Lys Pro Leu Val Val Thr Pro Gln Val Asn Tyr Thr Ala
    290                 295                 300

Tyr Glu Met Asp Arg Phe Phe Ala Asp Ile Gly Phe Ser Gln Gly Val
305                 310                 315                 320

Val Pro Tyr Lys Thr Arg Val Leu Pro Leu Thr Glu Glu Leu Met Thr
                325                 330                 335

Pro Gly Val Pro Val Thr Cys Ile Tyr Gly Arg Gly Val Asp Thr Pro
            340                 345                 350

Glu Val Leu Met Tyr Gly Lys Gly Gly Phe Asp Lys Gln Pro Glu Ile
        355                 360                 365

Lys Tyr Gly Asp Gly Asp Gly Thr Val Asn Leu Ala Ser Leu Ala Ala
    370                 375                 380

Leu Lys Val Asp Ser Leu Asn Thr Val Glu Ile Asp Gly Val Ser His
385                 390                 395                 400

Thr Ser Ile Leu Lys Asp Glu Ile Ala Leu Lys Glu Ile Met Lys Gln
                405                 410                 415

Ile Ser Ile Ile Asn Tyr Glu Leu Ala Asn Val Asn Ala Val Asn Glu
            420                 425                 430

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<211>  1998
<212>  DNA
<213>  Arabidopsis thaliana

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tttctttacc actctgttcc ggtcccggcg atgcttcaag ctccggagtc tccgggaact 240
cggttgagtc gagacggtgt caaggcgttt catccggtga ttcttgttcc ggggattgta 300
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gcatcagaac tgccttcctc acaactctct acgctaaacg tcaaggaact gtcaagagta 1260
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35 40 45

Ile Gly Tyr Leu Cys Thr Ala Trp Trp Leu Leu Leu Phe Leu Tyr His
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Ser Val Pro Val Pro Ala Met Leu Gln Ala Pro Glu Ser Pro Gly Thr
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Arg Leu Ser Arg Asp Gly Val Lys Ala Phe His Pro Val Ile Leu Val
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Pro Gly Ile Val Thr Gly Gly Leu Glu Leu Trp Glu Gly Arg Pro Cys
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Ala Glu Gly Leu Phe Arg Lys Arg Leu Trp Gly Ala Ser Phe Ser Glu
115 120 125

Ile Leu Arg Arg Pro Leu Cys Trp Leu Glu His Leu Ser Leu Asp Ser
130 135 140

Glu Thr Gly Leu Asp Pro Ser Gly Ile Arg Val Arg Ala Val Pro Gly
145 150 155 160

Leu Val Ala Ala Asp Tyr Phe Ala Pro Cys Tyr Phe Ala Trp Ala Val
165 170 175

Leu Ile Glu Asn Leu Ala Lys Ile Gly Tyr Glu Gly Lys Asn Leu His
180 185 190

Met Ala Ser Tyr Asp Trp Arg Leu Ser Phe His Asn Thr Glu Val Arg
195 200 205

Asp Gln Ser Leu Ser Arg Leu Lys Ser Lys Ile Glu Leu Met Tyr Ala
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Thr Asn Gly Phe Lys Lys Val Val Val Val Pro His Ser Met Gly Ala
225   230   235   240

Ile Tyr Phe Leu His Phe Leu Lys Trp Val Glu Thr Pro Leu Pro Asp
                245   250   255

Gly Gly Gly Gly Gly Gly Pro Gly Trp Cys Ala Lys His Ile Lys Ser
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Val Val Asn Ile Gly Pro Ala Phe Leu Gly Val Pro Lys Ala Val Ser
        275   280   285

Asn Leu Leu Ser Ala Glu Gly Lys Asp Ile Ala Tyr Ala Arg Ser Leu
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Ala Pro Gly Leu Leu Asp Ser Glu Leu Leu Lys Leu Gln Thr Leu Glu
305   310   315   320

His Leu Met Arg Met Ser His Ser Trp Asp Ser Ile Val Ser Leu Leu
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Pro Lys Gly Gly Glu Ala Ile Trp Gly Asp Leu Asp Ser His Ala Glu
            340   345   350

Glu Gly Leu Asn Cys Ile Tyr Ser Lys Arg Lys Ser Ser Gln Leu Ser
        355   360   365

Leu Ser Asn Leu His Lys Gln Asn Tyr Ser Leu Lys Pro Val Ser Arg
    370   375   380

Val Lys Glu Pro Ala Lys Tyr Gly Arg Ile Val Ser Phe Gly Lys Arg
385   390   395   400

Ala Ser Glu Leu Pro Ser Ser Gln Leu Ser Thr Leu Asn Val Lys Glu
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Phe Trp Ser Glu Tyr Asn Glu Met Ser Arg Glu Ser Ile Val Lys Val
        435   440   445

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Phe Ile Ala Pro Lys Met Met Arg Arg Ala Glu Ala His Phe Ser His
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Gly Ile Ala Asp Asp Leu Asp Asp Pro Lys Tyr Gly His Tyr Lys Tyr
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Trp Ser Asn Pro Leu Glu Thr Lys Leu Pro Glu Ala Pro Glu Met Glu
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Met Tyr Cys Leu Tyr Gly Val Gly Ile Pro Thr Glu Arg Ser Tyr Ile
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Tyr Lys Leu Ala Thr Ser Ser Gly Lys Cys Lys Ser Ser Ile Pro Phe
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Arg Ile Asp Gly Ser Leu Asp Gly Asp Asp Val Cys Leu Lys Gly Gly
545                 550                 555                 560

Thr Arg Phe Ala Asp Gly Asp Glu Ser Val Pro Val Ile Ser Ala Gly
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Phe Met Cys Ala Lys Gly Trp Arg Gly Lys Thr Arg Phe Asn Pro Ser
            580                 585                 590

Gly Met Asp Thr Phe Leu Arg Glu Tyr Lys His Lys Pro Pro Gly Ser
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Leu Leu Glu Ser Arg Gly Thr Glu Ser Gly Ala His Val Asp Ile Met
    610                 615                 620

Gly Asn Val Gly Leu Ile Glu Asp Val Leu Arg Ile Ala Ala Gly Ala
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Ser Gly Gln Glu Ile Gly Gly Asp Arg Ile Tyr Ser Asp Val Met Arg
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Met Ser Glu Arg Ile Ser Ile Lys Leu
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ggg 2223

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<212> DNA
<213> Schizosaccharomyces pombe

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<212> PRT
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Ser Glu Gln Pro Ser Ala Ser Glu Thr Gln Ser Val Ser Asn Lys Ser
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Arg Lys Ser Lys Phe Gly Lys Arg Leu Asn Phe Ile Leu Gly Ala Ile
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Leu Gly Ile Cys Gly Ala Phe Phe Phe Ala Val Gly Asp Asp Asn Ala
65 70 75 80

Val Phe Asp Pro Ala Thr Leu Asp Lys Phe Gly Asn Met Leu Gly Ser
85 90 95

Ser Asp Leu Phe Asp Asp Ile Lys Gly Tyr Leu Ser Tyr Asn Val Phe
100 105 110

Lys Asp Ala Pro Phe Thr Thr Asp Lys Pro Ser Gln Ser Pro Ser Gly
115 120 125

Asn Glu Val Gln Val Gly Leu Asp Met Tyr Asn Glu Gly Tyr Arg Ser
130 135 140

Asp His Pro Val Ile Met Val Pro Gly Val Ile Ser Ser Gly Leu Glu
145 150 155 160

Ser Trp Ser Phe Asn Asn Cys Ser Ile Pro Tyr Phe Arg Lys Arg Leu
165 170 175

Trp Gly Ser Trp Ser Met Leu Lys Ala Met Phe Leu Asp Lys Gln Cys
180 185 190

Trp Leu Glu His Leu Met Leu Asp Lys Lys Thr Gly Leu Asp Pro Lys
195 200 205

Gly Ile Lys Leu Arg Ala Ala Gln Gly Phe Glu Ala Ala Asp Phe Phe
210 215 220

Ile Thr Gly Tyr Trp Ile Trp Ser Lys Val Ile Glu Asn Leu Ala Ala
225 230 235 240

Ile Gly Tyr Glu Pro Asn Asn Met Leu Ser Ala Ser Tyr Asp Trp Arg
245 250 255

Leu Ser Tyr Ala Asn Leu Glu Glu Arg Asp Lys Tyr Phe Ser Lys Leu
260 265 270

Lys Met Phe Ile Glu Tyr Ser Asn Ile Val His Lys Lys Lys Val Val
275 280 285

Leu Ile Ser His Ser Met Gly Ser Gln Val Thr Tyr Tyr Phe Phe Lys
    290   295   300

Trp Val Glu Ala Glu Gly Tyr Gly Asn Gly Gly Pro Thr Trp Val Asn
305   310   315   320

Asp His Ile Glu Ala Phe Ile Asn Ile Ser Gly Ser Leu Ile Gly Ala
                325   330   335

Pro Lys Thr Val Ala Ala Leu Leu Ser Gly Glu Met Lys Asp Thr Gly
            340   345   350

Ile Val Ile Thr Leu Asn Ile Leu Glu Lys Phe Phe Ser Arg Ser Glu
        355   360   365

Arg Ala Met Met Val Arg Thr Met Gly Gly Val Ser Ser Met Leu Pro
    370   375   380

Lys Gly Gly Asp Val Ala Pro Asp Asp Leu Asn Gln Thr Asn Phe Ser
385   390   395   400

Asn Gly Ala Ile Ile Arg Tyr Arg Glu Asp Ile Asp Lys Asp His Asp
                405   410   415

Glu Phe Asp Ile Asp Asp Ala Leu Gln Phe Leu Lys Asn Val Thr Asp
            420   425   430

Asp Asp Phe Lys Val Met Leu Ala Lys Asn Tyr Ser His Gly Leu Ala
        435   440   445

Trp Thr Glu Lys Glu Val Leu Lys Asn Asn Glu Met Pro Ser Lys Trp
    450   455   460

Ile Asn Pro Leu Glu Thr Ser Leu Pro Tyr Ala Pro Asp Met Lys Ile
465   470   475   480

Tyr Cys Val His Gly Val Gly Lys Pro Thr Glu Arg Gly Tyr Tyr Tyr
                485   490   495

Thr Asn Asn Pro Glu Gly Gln Pro Val Ile Asp Ser Ser Val Asn Asp
            500   505   510

Gly Thr Lys Val Glu Asn Gly Ile Val Met Asp Asp Gly Asp Gly Thr
        515   520   525

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Leu Pro Ile Leu Ala Leu Gly Leu Val Cys Asn Lys Val Trp Gln Thr
    530                 535                 540

Lys Arg Phe Asn Pro Ala Asn Thr Ser Ile Thr Asn Tyr Glu Ile Lys
545                 550                 555                 560

His Glu Pro Ala Ala Phe Asp Leu Arg Gly Gly Pro Arg Ser Ala Glu
                565                 570                 575

His Val Asp Ile Leu Gly His Ser Glu Leu Asn Glu Ile Ile Leu Lys
            580                 585                 590

Val Ser Ser Gly His Gly Asp Ser Val Pro Asn Arg Tyr Ile Ser Asp
        595                 600                 605

Ile Gln Glu Ile Ile Asn Glu Ile Asn Leu Asp Lys Pro Arg Asn
    610                 615                 620


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<212>  DNA
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SUBSTITUTE SHEET (RULE 26)

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            20                  25                  30

Leu Lys Arg Ser Gly Lys Lys Asp Lys Ser Lys Asn Tyr Ser Cys Leu
        35                  40                  45

Asp Asn Cys Cys Trp Phe Val Gly Cys Val Cys Thr Ala Trp Trp Leu
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Gly Gly Gly Pro Asp Trp Cys Ala Lys His Ile Lys Ala Val Met Asn
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Ser Ala Glu Ala Lys Asp Ile Ala Ser Ala Arg Ala Leu Ala Pro Asn

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Val Leu Asp Ser Asp Leu Phe Gln Ile Gln Thr Leu Gln His Leu Met
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Leu Phe Val Tyr His Cys Leu Pro Ala His Leu Ala Gly Phe Lys Gly
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Pro Glu Pro Pro Gly Val Arg Leu Lys Asn Glu Gly Leu Thr Pro Leu
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His Pro Val Val Leu Val Pro Gly Ile Val Thr Gly Gly Leu Glu Leu
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Trp Glu Gly Gln Pro Cys Ser His Gly Leu Phe Arg Lys Arg Leu Trp
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Gly Gly Ser Phe Thr Glu Ile Leu Glu Arg Pro Leu Cys Trp Leu Glu
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His Leu Ser Leu Asp Asn Glu Thr Gly Leu Asp Pro Pro Gly Ile Arg
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Val Arg Pro Val Pro Gly Leu Val Ala Ala Asp Tyr Phe Ala Pro Gly
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Tyr Phe Val Trp Ala Val Leu Ile Glu Asn Leu Ala Lys Ile Gly Tyr
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Glu Gly Lys Asn Met Tyr Met Ala Ala Tyr Asp Trp Arg Leu Ser Phe
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Gln Asn Thr Glu Val Arg Asp Gln Ala Leu Ser Arg Leu Lys Ile Asn
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Val Ile Ser Leu Leu Pro Lys Gly Gly Asp Thr Ile Trp Gly Asp Leu
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Asp Ser Ser Pro Glu Asp Phe Glu Thr Glu Asn His Gly Lys Ile Asn
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Asn Thr Lys Pro Gln Phe Val Met Lys Gln Glu Thr Lys Tyr Gly Arg
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Thr Asn His Asp Leu Gln Lys Asp Asn Leu Phe Asp Thr Gly Ala Asn
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Cys Gly Glu Ser Trp Ser Glu Tyr Gly Lys Ile Ser Lys Glu Ser Ile
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Ile Lys Leu Ala Asp Asn Lys Ala Tyr Thr Ala Gly Thr Leu Ile Asp
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Leu Leu Arg Phe Val Ala Pro Lys Thr Met Lys Arg Ala Glu Ala His
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Phe Ser His Gly Ile Ala Asp Asp Leu Asp Asp Pro Lys Tyr Asn His
                485                 490                 495

Tyr Lys Tyr Trp Ser Asn Pro Leu Glu Thr Lys Leu Pro Asp Ala Pro
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Asn Met Glu Ile Phe Ser Leu Tyr Gly Val Gly Leu Ser Thr Glu Arg
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Ser Ala Gly Phe Met Cys Ala Lys Gly Trp Lys Gly Lys Thr Arg Phe
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Asn Pro Ser Gly Ser Glu Thr Tyr Ile Arg Glu Tyr Lys His Lys Ala
        595                 600                 605

Pro Gly Ser Leu Leu Glu Gly Arg Gly Leu Glu Ser Gly Ala His Val
    610                 615                 620

Asp Ile Leu Gly Asn Val Ala Leu Ile Glu Asp Val Leu Arg Val Ala
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Ala Gly Ala Ser Gly Val Glu Ile Gly Gly Asp Arg Ile Tyr Ser Asp
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Ile Leu Lys Met Ala Asp Arg Val Asn Ile Lys Leu
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 03/00870

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 9/10, A61K 38/45

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI DATA, EPO-INTERNAL, BIOSIS, MEDLINE, CHEM. ABS DATA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6300487 A (LEUNG ET AL), 9 October 2001 (09.10.01), column 10, line 1 - line 40 --	1-35
A	WO 0060095 A2 (BASF PLANT SCIENCE GMBH), 12 October 2000 (12.10.00) --	1-35
A	US 5324663 A (LOWE), 28 June 1994 (28.06.94), column 19, line 49 - line 58 -- --------	1-35

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 October 2003	17-10-2003
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz/EÖ Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE03/00870

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 6-7
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see next sheet*

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet**

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-5 (partly), 6-7, 12-35 (partly)

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

*

Present claims 6-7 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts related to the compounds disclosed in Example 1, i.e. the nucleotide sequences encoding the amino acid residues 98-661, 170-661, and 190-661 of SEQ ID NO:1. Further, the variants having nucleotide sequence residues 238-288 or 1-291 deleted from SEQ ID NO:1 have been possible to search specifically.

**

The International Search Authority (ISA) considers that there are 8 inventions covered by the claims indicated as follows:

1.Claims 1-5 (partly), 6-7, 12-35 (partly): A nucleotide sequence encoding an improved active membrane independent acyltransferase polypeptide wherein from 1-291 nucleotide sequence residue(s) of the 5'-end has/have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:1 or wherein at least the nucleotide sequence residues 238-288 have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID No:1.

2. Claims 1-5 (partly), 8-9 (partly), 12-35 (partly): A nucleotide sequence encoding an improved active membrane independent acyltransferase polypeptide wherein from 1-219 nucleotide sequence residue(s) of the 5'-end has/have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO: 4 or wherein at least the nucleotide sequence residues 142-210 have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:4.

../..

3. Claims 1-5 (partly), 8-9 (partly), 12-35 (partly): A nucleotide sequence encoding an improved active membrane independent acyltransferase polypeptide wherein from 1-87 nucleotide sequence residue(s) of the 5´-end has/have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO: 8 or wherein at least the nucleotide sequence residues 19-87 have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:8.

4. Claims 1-5 (partly), 8-9 (partly), 12-35 (partly): A nucleotide sequence encoding an improved active membrane independent acyltransferase polypeptide wherein from 1-87 nucleotide sequence residue(s) of the 5´-end has/have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO: 10 or wherein at least the nucleotide sequence residues 19-87 have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:10

5. Claims 1-5 (partly), 8-9 (partly), 12-35 (partly): A nucleotide sequence encoding an improved active membrane independent acyltransferase polypeptide wherein from 1-190 nucleotide sequence residue(s) of the 5´-end has/have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO: 12 or wherein at least the nucleotide sequence residues 130-190 have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:12.

6. Claims 1-5 (partly), 10-35 (partly): A nucleotide sequence encoding an improved active membrane independent acyltransferase polypeptide wherein from 1-228 nucleotide sequence residue(s) of the 5´-end has/have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO: 16 or wherein at least the nucleotide sequence residues 169-228 have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:16.

7. Claims 1-5 (partly), 10-35 (partly): A nucleotide sequence encoding an improved active membrane independent acyltransferase polypeptide wherein from 1-219 nucleotide sequence residue(s) of the 5´-end has/have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO: 18 or wherein at least the nucleotide sequence residues 151-219 have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:18.

.../...

8. Claims 1-5 (partly), 10-35 (partly): A nucleotide sequence encoding an improved active membrane independent acyltransferase polypeptide wherein from 1-261 nucleotide sequence residue(s) of the 5'-end has/have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO: 20 or wherein at least the nucleotide sequence residues 193-261 have been deleted and/or substituted from the nucleotide sequence shown in SEQ ID NO:20.

The ISA has carried out a partial search which relates to invention 1 mentioned above. The present application has been considered to contain 8 inventions which are not linked such that they form a single general inventive concept, as required by Rules 13.1, 13.2 and 13.3 PCT for the following reasons:

The prior art has been identified as US 6300487 (D1). D1 concerns a lysophosphatidic acid acyltransferase which catalyzes the acylation of lysophosphatidic acid to the phospholipid phosphatidic acid. It is disclosed that the presence of transmembrane sequences is often deleterious when a recombinant protein is synthesized in many expression systems due to the production of insoluble aggregates. Deletion of one or more transmembrane sequences is therefore suggested in D1. Transmembrane sequences are readily detected by the use of standard sequence analysis software (see column 10, lines 1-40 in D1).

Invention 1 of the present application differs from what is known from D1 in that the nucleotide sequence encoding the original acyltransferase is represented by SEQ ID NO:1. The problems to be solved by Invention 1 is considered to be to provide further membrane-independent acyltransferases.

Inventions 2-8 differ from what is known from D1 in that the nucleotide sequence encoding the original acyltransferase is represented by SEQ ID NO:s 4, 8, 10, 12, 16, 18 and 20 respectively. The problems to be solved by Inventions 2-8 are considered to be to provide further membrane-independent acyltransferases.

.../...

Thus, the different inventions provides membrane-independent acyltransferases represented by different nucleotide sequences encoding them. No single general inventive concept has been found between the nucleotide sequences encoding the different acyltransferases.

In conclusion, no technical relationship among the claimed inventions involving one or more of the same or corresponding "special technical features", i.e features that define a contribution which each of the inventions makes over the prior art, has been found (PCT Rule 13.2).

INTERNATIONAL SEARCH REPORT
Information on patent family members

06/09/03

International application No.
PCT/SE 03/00870

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6300487 A	09/10/01	US 2002156262 A	24/10/02
		US 6060263 A	09/05/00
		US 6136964 A	24/10/00
		WO 0037655 A	29/06/00
WO 0060095 A2	12/10/00	AU 3814700 A	23/10/00
		BR 0009510 A	23/04/02
		CA 2366187 A	12/10/00
		CN 1362994 T	07/08/02
		CZ 20013529 A	13/02/02
		EP 1165803 A	02/01/02
		HU 0200480 A	29/07/02
		IL 145307 D	00/00/00
		JP 2002541783 T	10/12/02
		NO 20014716 A	28/11/01
		SK 13872001 A	04/06/02
		TR 200102859 T	00/00/00
US 5324663 A	28/06/94	AT 188999 T	15/02/00
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		DE 69131925 D	00/00/00
		EP 0515536 A,B	02/12/92
		JP 5504480 T	15/07/93
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		US 5595900 A	21/01/97
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		US 6268193 B	31/07/01
		US 2002102688 A	01/08/02
		WO 9112340 A	22/08/91
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		EP 0654082 A	24/05/95
		IL 106416 D	00/00/00
		JP 8505041 T	04/06/96
		NZ 255056 A	24/04/97
		NZ 314354 A	28/07/98
		PH 30252 A	05/02/97
		WO 9402616 A	03/02/94
		ZA 9305237 A	18/03/94